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DISSERTATION THESIS

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Clinical biochemistry

The effect on bone metabolism of selected substances affecting the central nervous system

Vliv vybraných látek ovlivňujících centrální nervový systém na kostní metabolismus

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Hradec Králové, 2015 Defence on:.....

Declaration

I declare hereby that this dissertation thesis is my own original work and that I have indicated by references all used information sources. I also agree to deposit my dissertation in the Medical Library of the Charles University in Prague, Faculty of Medicine in Hradec Králové and to make it available for study and educational purposes provided that anyone who will use it for his/her publication or lectures is obliged to refer to or cite my work properly.

I give my consent to making available the electronic version of my dissertation in the information system of Charles University in Prague.

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Abbreviation

AED	antiepileptic drug
BALP	bone alkaline phosphatase
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BMP-2	bone morphogenetic protein
BMU	basic multicellular unit
CBZ	carbamazepine
CNS	central nervous system
CRP	C-reactive protein
CTX-I	carboxy-terminal cross-linking telopeptide of type I collagen
CYP450	cytochrome P450
DEXA	dual energy x-ray absorptiometry
ELISA	Enzyme-linked immunosorbent assay
ESI	electrospray ionization
GABA	gamma-aminobutyric acid
GI	gastrointestinal tract
HPLC	high-performance liquid chromatography
HPLC-MS	high-performance liquid chromatography – mass spectrometry
IGF-1, 2	insulin-like growth factor 1, 2
IGFBP	insulin-like growth factor – binding protein
IL 6	interleukin 6
LCM	lacosamide
LEV	levetiracetam

LF	left femur
LTG	lamotrigine
MDD	major depressive disorder
MIRTA	mirtazapine
NaSSA	noradrenergic and specific serotonergic antidepressant
OPG	osteoprotegerin
ORX	orchidectomy
P1CP	carboxy-terminal propeptide of procollagen type 1
P1NP	amino-terminal propeptide of procollagen type 1
PB	phenobarbital
PBS	phosphate buffer
PGE2	prostaglandin E2
PHT	phenytoin
PRM	primidone
PTH	parathormone
RANK	activator of nuclear factor kappa-B ligand
RANKL	receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt-related transcription factor 2
RF	right femur
SLD	standard laboratory diet
SNRI	serotonin and noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
SVP	sodium valproate
TCA	tricyclic antidepressant
TNRF	tumor necrosis factor receptor
TPM	topiramate

TRA	trazodone
TRANCE	tumor necrosis factor – related activation induced cytokine
VENLA	venlafaxine
VPA	valproate
5-HTT	serotonin transporter

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1. INTRODUCTION

Bone health is maintained by a balanced remodelling process that ensures the continual replacement of old bone, weakened by microfractures, with new bone. This is a coupled process involving bone resorption by osteoclasts and new bone formation by osteoblasts *(McCormick RK; 2007).* The equilibrium between bone formation and resorption is important, because an imbalance of bone resorption and formation results in several bone diseases. For example, excessive resorption by osteoclasts without the corresponding amount of new-formed bone by osteoblasts contributes to bone loss and osteoporosis *(Florencio-Silva R, et al., 2015).*

The past decade has witnessed a remarkably increased awareness of osteoporosis as a major health problem that is associated with profound socio-economic consequences *(Dunitz M, 2001).* Osteoporosis is a systemic skeletal disorder and is a result of loss of skeletal mass. The term “osteoporosis” is derived from the Greek language: osteon means bone, and poros is a small hole. Thus the term “osteoporosis” is quite descriptive of the changes in bone tissue that can be observed in this generalized skeletal disease *(Dunitz M, 1998).* In the European Union (EU 27), an estimated 5.5 million men and 22 million women have osteoporosis. About 6.6% of men and 22.1% of women aged 50 years and older are affected. Osteoporosis is characterized by low

bone mass, microarchitectural deterioration of bone tissue and an increase in bone fragility and susceptibility to fracture *(Hammad LF, 2015; Schürer C, et al 2015)*. Osteoporotic fractures are a significant cause of morbidity and mortality *(Wheater G, 2013).* There have been impressive advances in understanding the epidemiology and pathogenesis of osteoporosis and its associated fractures, in the application of physical and biochemical methods to its diagnosis and evaluation, and in the therapeutic approaches to prevention and treatment of postmenopausal and other forms of osteoporosis *(Dunitz M, 2001).* The longterm use of drugs such as antiepileptics and antidepressants could affect the onset of osteoporosis.

2. BONE METABOLISM

Bone is essential for providing skeletal strength and vital organ protection, and is a mineral reservoir for calcium and a site for immune-cell development. Bone cell homeostasis is maintained by the balanced functions of primarily two cell-types: osteoblasts, which build bone, and osteoclasts which resorb bone *(Walsh MC, et al., 2014).* There exist two types of bone tissue in the adult, cortical or compact bone, and spongy or cancellous bone. Most bones have an outer cortical casing comprising an outer (periosteal) and inner surface which encloses the cancellous bone and marrow space. Bone comprises an organic matrix, a mineral phase and bone cells. The majority of the matrix is composed

of collagen fibres, which account for 90% of skeletal weight in the adult. Bone is constantly being broken down and rebuilt in a process called remodelling. The cellular link between bone-resorbing cells – osteoclasts, and bone-forming cells – osteoblasts, is known as coupling *(Marcus R,et al., 1995; Kanis JA, 1994).*

2.1. Bone formation and resorption

Bone formation takes place in the organism not only during embryonic development and growth but throughout life in the processes of normal bone remodelling and fracture repair. In the mature adult, skeletal size is neither increasing nor decreasing. Despite this, bone is continuously being turned over, so that the net activity of bone-resorbing cells equals the net activity of bone-forming cells. In the adult, this activity is largely accounted for by bone remodelling. Bone remodelling is responsible for removal and repair of damaged bone to maintain the integrity of the adult skeleton and mineral homeostasis. *(Kanis JA, 1994; Marcus R, et al 1995; Dunitz M, 2001; Ragatt LJ and Partridge NC; 2010)*. Bone remodelling occurs over several weeks and is performed by clusters of bone-resorbing osteoclasts and bone-forming osteoblasts arranged within temporary anatomical structures known as “basic multicellular units” (BMUs). Traversing and encasing the BMU is a canopy of cells that creates a bone-remodelling compartment. An active BMU consists of

a leading front of osteoclasts and a tail portion comprising osteoblasts. *(Ragatt LJ and Partridge NC; 2010)*.

Osteoblasts are differentiated from osteoprogenitor cells. Osteoblastic cells comprise a diverse population of cells that include immature osteoblast lineage cells and differentiating and mature matrix-producing osteoblasts *(Ragatt LJ and Partridge NC; 2010)*. A mature osteoblast is derived from the preosteoblast and expresses all of the differential functions required to synthesize bone. The osteoblast is the cell responsible for the synthesis of collagen and other bone proteins. It also has an important role in the subsequent mineralization of the matrix which leads to the final stage of osteoblast differentiation. The most mature or terminally differentiated cells of the osteoblast lineage are *osteocytes*, which not capable of cell division. They are trapped within the bone matrix during the process of *bone formation*, and are interconnected to osteoblast and other osteocytes by fine intercellular projections running within bone canaliculi *(Kanis JA, 1994; Marcus R, 1995).* They are critical for maintaining fluid flow through the bone, and changes in this fluid flow may provide the signal for the cellular response to mechanical forces (Raisz 1999). Data have been obtained that support the idea that osteocytes initiate and direct the subsequent remodelling process that repairs damaged bone *(Ragatt LJ and Partridge NC; 2010).* Bone *lining cells* are usually designated as part of the osteoblast lineage and are believed to be derived from osteoblasts that have ceased their activity and flattened out on bone surfaces

that are undergoing neither formation nor resorption. They have fewer organelles than the active osteoblast, further suggesting that they may be largely a selective barrier between bone and other extracellular compartments, and contribute to mineral homeostasis by regulating the fluxes of calcium and phosphate in and out of bone fluids *(Marcus R, 1995; Kanis JA, 1994; Raisz LG,1999; Ragatt LJ and Partridge NC; 2010)*.

The other major cell type found in bone is the *osteoclast*. This is a multinucleated cell which is responsible for bone resorption. The precursors of osteoclasts are hemopoietic mononuclear cells which are resident in the bone marrow. The functional role of the osteoclast is to resorb bone, a composite matrix consisting of both inorganic and organic elements. The initial step in bone resorption is attachment of the cell to the matrix by attaching on to a bone surface and secreting acid and lysosomal enzymes into the space provided between its apical surface and the mineralized bone surface. The surface of the osteoclast at this interface is ruffled by cytoplasmic extensions that infiltrate the resorbing bone surface *(Kanis JA, 1994; Marcus R, 1995; Dunitz M, 1998).*

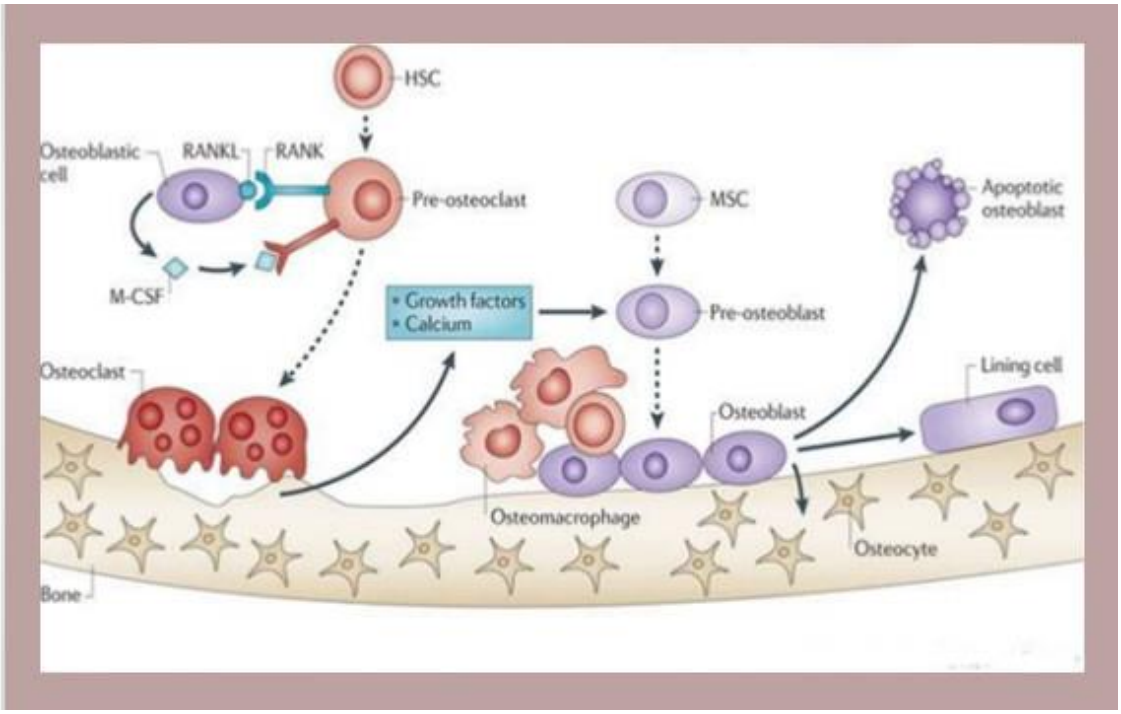

Figure 1. Bone remodelling. Modified by *Weilbaecher KN, et al., 2011.*

2.2. Steps of remodelling

The bone remodelling cycle involves a complex series of sequential steps that are highly regulated *(Kanis JA, 1994).* Bone remodelling can be divided into the following six phases, namely, quiescent, activation, resorption, reversal, formation, and mineralization (Fig. 2) *(Kini U end Nandeesh BN, 2012).*

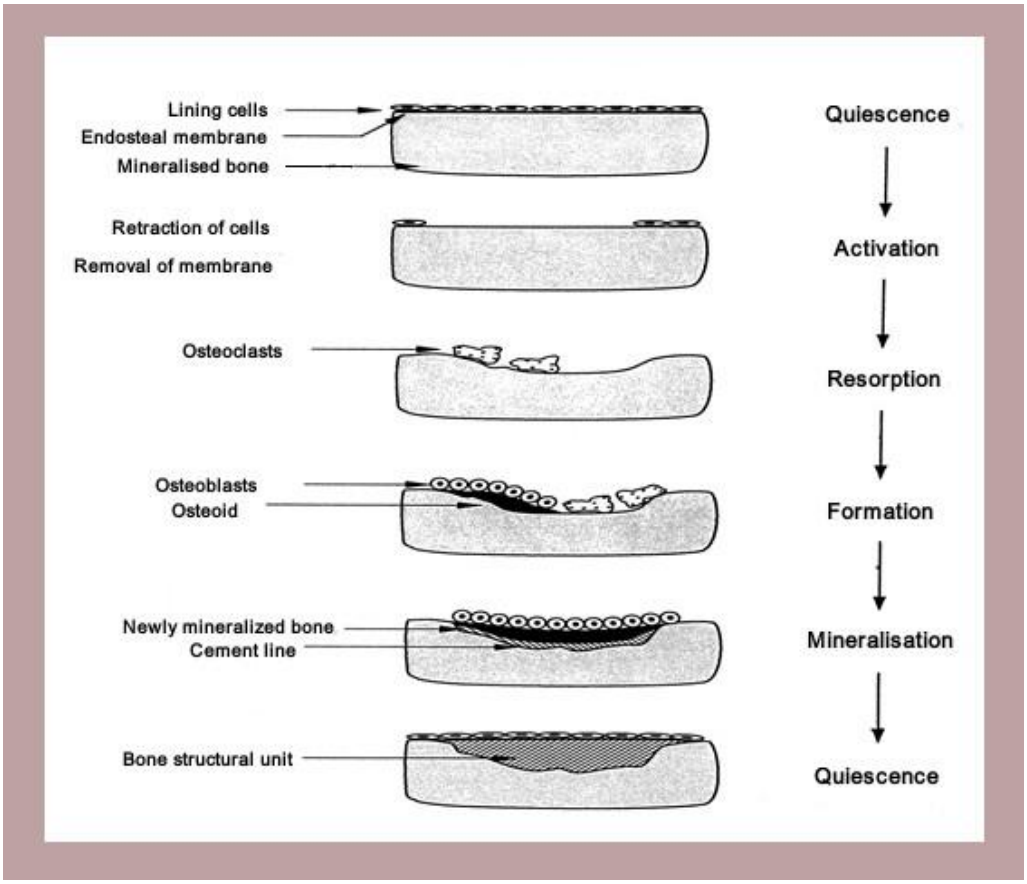

Figure 2. Steps of bone remodelling. Modified by *Compston JE, 2001.*

The first step of bone remodelling is the focal attraction of osteoclasts to the quiescent bone surface and is termed *activation*. The term refers to the event and not to the activity of the osteoclasts themselves. In health, an activation occurs every 10 seconds or so, and its frequency will largely determine the number of new remodelling sites present on bone tissue. This phase is dependent on the effects of local and systemic factors on mesenchymal cells of the osteoblast lineage *(Kanis JA, 1994; Raisz LG,1999).*

The second phase of remodelling is *resorption*, during which the osteoclasts cut an erosion cavity to a depth of 40 - 60 um over 4-12 days.

Thereafter, the multinucleated cells disappear and are replaced by mononuclear cells, which remove collagen remmants and prepare the bone surface for subsequent osteoblast-mediated bone formation. Over the next 7-10 days a layer of cement substance is deposited which is rich in proteoglycans, glycoproteins and acid phosphatase but poor in collagen. Once the osteoclasts have completed their work of bone removal, there is a *reversal phase* during which mononuclear cells are seen on the bone surface. They may complete the resorption process and produce the signals that initiate formation *(Kanis JA, 1994; Raisz LG,1999;* R*agatt LJ and Partridge NC, 2010)*.

After resorption and the reversal phase follows the process termed *coupling*, which coordinates this transition and directs bone formation precisely to sites of bone resorption. Coupling attracts osteoblasts to the eroded surface where they synthesize an osteoid matrix. The amount of new bone formed is also dependent on the number and activity of the osteoblasts *(Kanis JA, 1994; Ragatt LJ and Partridge NC, 2010).*

Once osteoclasts have resorbed a cavity of bone, they detach from the bone surface and are replaced by cells of the osteoblast lineage which in turn initiate bone *formation (Kini U end Nandeesh BN, 2012).* Osteoblasts form a sheet of cells within the resorption cavity and synthesize layers of osteoid matrix which comprise unmineralized bone tissue and other matrix proteins. Matrix synthesis is rapid during the initiation of the formation phase. The newly formed

osteoid has a lamellated arrangement of collagen due to changes in the orientation of collagen bundles. In cancellous bone the lamellae are usually parallel to the trabeculae *(Kanis JA, 1994).*

When an equal quantity of resorbed bone has been replaced, the remodelling cycle concludes. The termination signal(s) that inform the remodelling machinery to cease work are largely unknown, although a role for osteocytes is emerging. Sclerostin expression, loss of which was the trigger to initiate osteoblastic bone formation, probably reverts to normal toward the end of the remodelling cycle *(Ragatt LJ and Partridge NC; 2010).* The process of mineralization begins 30 days after deposition of the osteoid, ending at 90 days in trabecular and at 130 days in cortical bone *(Kini U end Nandeesh BN, 2012).* The mineral phase of bone is mainly calcium, phosphate and carbonate (10:6:1) arranged as crystals predominantly in the form of hydroxyapatite. Crystals of hydroxyapatite are elongated and hexagonal in shape, conforming closely to the orientation of the collagen fibres. They also contain other ions including sodium, magnesium and fluoride. The delay between the onset of matrix synthesis and the start of mineralization accounts for the appearance of osteoid in normal bone, during which maturation of osteoid occurs. The first step in calcification is thought to take place in or around small membrane-bound vesicles, rich in bone alkaline phosphatase (BALP) *(Kanis JA, 1994).* Following mineralization, mature osteoblasts undergo apoptosis, revert back to a bone-lining phenotype or become embedded in the mineralized matrix and differentiate into

osteocytes. The resting bone surface environment is re-established and maintained in quiescent phase until the next wave of remodelling is initiated *(Ragatt LJ and Partridge NC, 2010; Kini U end Nandeesh BN, 2012).*

2.3. Regulation of bone remodelling

The balance between bone resorption and formation is influenced by such interrelated factors *(Kini U end Nandeesh BN, 2012)*. The effects of calcium-regulating hormones on this remodelling cycle subserve the metabolic functions of the skeleton. Other systemic hormones control overall skeletal growth. The responses to changes in mechanical force and repair of microfractures, as well as the maintenance of the remodelling cycle, are determined locally by cytokines, prostaglandins, and growth factors. Interactions between systemic and local factors are important in the pathogenesis of osteoporosis (*Raisz LG,1999).*

- **Estrogens**

Estrogen plays crucial roles for bone tissue homeostasis *(Florencio-Silva R, et al., 2015)*. Its loss in postmenopausal women leads to osteoporosis characterized by low bone mass, altered bone microarchitecture, and increased risk of fracture *(Emerton KB, et al., 2010).* Although the mechanisms by which

estrogen acts on bone tissue are not completely understood *(Florencio-Silva R, et al., 2015)*, several studies have shown that estrogen maintains bone homeostasis by inhibiting osteoblast and osteocyte apoptosis and preventing excessive bone resorption *(Kousteni S, et al., 2002; Emerton KB, et al., 2010)* . It is now recognized that one of the main mechanisms by which estrogen deficiency causes bone loss is by stimulating formation, a process induced by stimulation of precursor simultaneously by M-CSF, TNF and RANKL *(Roggia C, et al., 2001).* It follows that estrogens interfere with production of RANKL/OPG *(Macari S, et al., 2015).*

- Androgens

Androgens have an anabolic effect on bone through the stimulation of the osteoblast receptors *(Kanis JA, 1994).* They have direct receptor-mediated effects on bone cells and probably effect skeletal metabolism in a manner comparable to estrogen. In addition, the anabolic effect of testosterone on muscle mass is probably one of the mechanisms responsible for the greater skeletal mass in men than woman at maturity. Unlike women, men do not undergo a natural menopause, and spermatogenesis in contrast to ovulation occurs throughout life, although it may decline with age. The production of gonadal steroids is also sustained, but may wane in the very old, causing accelerated loss of bone *(Kini U end Nandeesh BN, 2012)*.

- **IGF - I and II (Insulin-like Growth Factor I and II)**

These are polypeptides similar to insulin; they are synthesized by the liver and by osteoblasts, and found in high concentrations in the osteoid matrix *(Dunitz M, 2001).* They increase the number and of the osteoblasts, stimulating collagen synthesis. They circulate linked to IGF-binding proteins (IGFBP), which in turn can exercise stimulatory or inhibitory effects on bone. IGF synthesis is regulated by local growth factors and hormones; thus growth hormone, estrogens, and progesterone increase its production, while the glucocorticoids inhibit it. They also mediate in the osteoblast-osteoclast interaction and actively participate in bone remodelling. Insulin-like growth factor (IGF I), formerly known as somatomedin C, stimulates the replication of bone cells and chondrocytes, and increases production of matrix constituents *(Kini U end Nandeesh BN, 2012)*. Both IGF I and IGF II are produced within bone itself, and their activity is modulated by specific binding proteins which are also under endocrine control. There may be important species differences in the regulation by IGFs, with IGF II dominating in human bone, whilst IGF I is the major endogenous IGF in rat bone *(Dunitz M, 2001).*

- **RANK/RANKL/OPG protein system**

The RANKL/RANK/OPG pathway plays key roles in the development of osteoclasts and the regulation of the immune system *(Nagy V and Penniger JM, 2015).* This system consists of the cytokine receptor activator of nuclear factor

kappa-B ligand (RANKL), also identified as TNF-related activation-induced cytokine (TRANCE); its signalling receptor, receptor activator of NF-κB (RANK); and the soluble decoy receptor osteoprotegerin (OPG) *(Walsh MC end Choi Y, 2014).* OPG is a member of the tumor necrosis factor receptor (TNFR) superfamily, and acts by competing with receptor activator of nuclear factor kappa-B (RANK), which is expressed on osteoclasts for specifically binding to RANKL. Transgenic mice that overexpress OPG demonstrated an increase in bone density, whilst OPG knockout mice exhibit profound osteoporosis. These findings show a crucial role for OPG in the maintenance of bone mass, but it seems that OPG may neutralize a TNF-related factor that could stimulate osteoclast development. This factor was identified as RANKL, which was originally called TRANCE (TNF-related activation-induced cytokine) *(Maxhimer JB et al., 2015; Schoppet M et al., 2002)*. OPG expression is regulated both positively (e.g., TGF-β, IL-1, TNF, estrogen, and Wnt ligands) and negatively (e.g., prostaglandin E2 (PGE2) and glucocorticoids) by a wide array of factors, most of which are associated with bone homeostasis *(Walsh MC end Choi Y, 2014).* RANKL is produced by osteoblastic lineage cells and promotes osteoclast activation leading to enhanced bone resorption and bone loss. OPG, which is secreted by osteoblastic cells, prevents RANKL interaction and subsequent stimulation with its receptor, RANK *(Schoppet M et al., 2002)*.

- **BMP**

The so-called bone morphogenetic proteins (BMPs) are factors present in demineralized bone matrix which can induce the formation of new cartilage and bone when implanted into various non-skeletal sites in vivo *(Dunitz M, 2001).* BMPs are included in the TGF-β family. They form a group of 15 proteins able to achieve the transformation of connective tissue into bone tissue, for which they are considered osteoinductive. They stimulate the differentiation of the stem cells toward different cell lines (adipose tissue, cartilage, and bone). They strongly promote osteoblastic differentiation and are believed to inhibit osteoclastogenesis in addition to stimulating osteogenesis *(Kini V end Nandeesh BN, 2012).* They are known to be important in regulating the differentiation of skeletal and other tissues, and may eventually be of therapeutic use, e.g. to promote repair of fractures and skeletal defects *(Dunitz M, 2001).*

- **Sclerostin**

Sclerostin is a 190-amino acid secreted glycoprotein made predominantly by osteocytes, but also by cementocytes and mineralized hypertrophic chondrocytes *(Shah AD, et al., 2015).* Sclerostin is produced by osteocytes and inhibits osteoblast differentiation and bone formation via the Wnt signaling pathway *(Bhattoa HP, et al., 2013).* Wnt signaling is crucial to both bone development and the regulation of bone mass. Wnt signaling in bone leads to

osteoblast differentiation, proliferation, function and survival, and hence to increased bone mass *(Shah AD, et al., 2015).* Upregulation of sclerostin is associated with decreased osteogenesis and bone mass *(Compton JT and Lee YF, 2014).*

2.3.1. Bone markers

Biochemical markers of bone metabolism provide dynamic information about the turnover of osseous tissue. They can be broadly classified as reflecting either bone formation or bone resorption *(Kini V end Nandeesh BN, 2012).* Markers of bone formation are either by-products of active osteoblasts expressed during the various phases of their development or osteoblastic enzymes.

- BALP = Bone alkaline phosphatase

The level of alkaline phosphatase in serum has been used for more than 50 years to monitor bone metabolism and is still the most frequently used marker. Alkaline phosphatase is an ectoenzyme anchored to the cell surface of osteoblasts and other cells *(Kini V end Nandeesh BN, 2012).* The serum concentration of BALP reflects the cellular activity of osteoblasts. Although its exact function in cells is presently unknown, its primary physiological role in

bone is associated with calcification of the skeleton and bone formation *(Dunitz M, 2001).*

- **P1NP = Type I procollagen N-terminal propeptide**

P1NP has several functional advantages and has been recommended by the Bone Marker Standards Working Group; it has low interindividual variability and is relatively stable at room temperature *(Wheater G, et al., 2013).* In bone, collagen is synthesised by osteoblasts in the form of pre-procollagen. These precursor molecules are characterised by short terminal extension-peptides: the amino (N-) terminal propeptide (P1NP) and the carboxy (C-) terminal propeptide (P1CP). After secretion into the extracellular space, the globular trimeric propeptides are enzymatically cleaved and liberated into the circulation. P1CP has a MW of 115 kDa and is stabilised by disulphide bonds. It is cleared by liver endothelial cells via the mannose receptor and therefore has a short serum half-life of 6–8 minutes. P1NP has a MW of only 70 kDa, is rich in proline and hydroxyproline, and is eliminated from the circulation by liver endothelial cells by the scavenger receptor *(Seibel MJ, 2005).* The serum concentration of P1NP also reflects changes in the synthesis of new collagen, both by osteoblasts in bone and by fibroblasts in other connective tissues *(Dunitz M, 2001).* P1NP is cleared by liver endothelial cells via a macrophage receptor species, the scavenger receptor, that recognises and endocytoses modified proteins. P1NP is released as a trimeric structure, but is rapidly broken down to a monomeric

form by thermal degradation *(Wheater G, et al., 2013).*

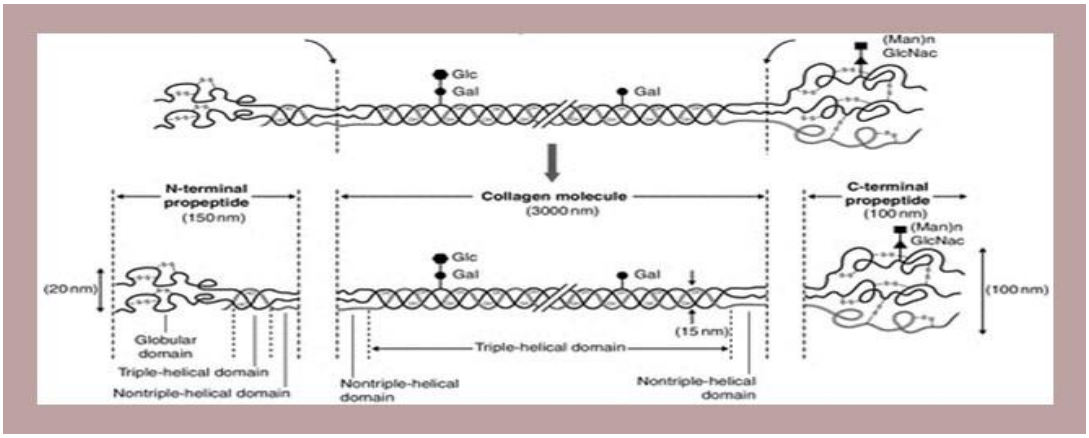

Figure 3. Schematic representation of the structure of the procollagen molecule.

- CTX

Type I collagen represents more than 90% of the organic matrix of bone. The modern markers of bone resorption, including the C– and N–telopeptides and pyridinoline cross-links, represent a vast improvement over the older biomarkers in specifity and sensitivity for mature bone.

3. EPILEPSY

Epilepsy is one of the most common neurological disorders of the brain. Worldwide, epilepsy affects almost 70 million people. One in every ten people will have at least one epileptic seizure during a normal lifespan, and a third of these will develop epilepsy *(Engel J and Pedley TA, 2008; Zhaoxia LI, et al., 2014)*. The disease is often chronic, and lifelong treatment may be required *(Svalheim S, at al., 2011).* Fracture rates are increased in patients with epilepsy. Although this increase may in part be secondary to seizure activity, the effects of AEDs on bone also contribute *(Engel J and Pedley TA, 2008).* AEDs are widely used and prescribed as standard treatment not only for epilepsy, but for a variety of non-epileptic conditions as well, mainly bipolar spectrum disorders and chronic pain states *(Reimers A, 2014).*

Antiepileptic drugs (AEDs) may alter bone mineral metabolism and may compromise bone health, especially in patients who have taken AEDs for a longer period *(Levy RH, et al., 2002).* A number of theories have been proposed to explain why AEDs affect bone, but none explains all the reported effects *(Svalheim S, at al., 2011).* Cytochrome P450 enzyme-inducing AEDs are those most commonly associated with negative impact on bone, but studies suggest such effect also with valproate. Data on bone-specific effects of newer AEDs are limited *(Pack A, 2008).*

Patients with epilepsy have a 2-6 times greater risk of bone fractures compared with the general population *(Svalheim S, at al., 2011).* Some fractures are caused by seizure-related injuries, or they may be associated with the osteopenic effect of reduced physical activity in patients with epilepsy. The risk of developing osteoporosis should be taken into consideration in the selection of an AED. Bone loss can occur slowly and asymptomatically, and it is important to manage it pre-emptively and thus help prevent fractures *(Svalheim S, at al., 2011).* It should be borne in mind that AEDs are used not only to treat epilepsy but also for other conditions such as headache and neuropathic pain *(Beniczky SA, et al., 2012).*

A large number of AEDs are available *(Reimers A, 2014).* The older generation of enzyme-inducing AEDs such as phenytoin (PHT), phenobarbitone, primidone, and carbamazepine have been frequently associated with accelerated bone loss, resulting from hepatic induction of cytochrome P450 (CYP450) hydroxylase enzymes causing catabolism of vitamin D to inactive metabolites. This would lead to an increase in parathyroid hormone levels, required for the body to convert more vitamin D into its active forms, and this increase in PTH would then cause an increase in bone turnover, with resultant bone loss over time. However, non-enzyme-inducing AEDs such as sodium valproate (SVP) are known to be also associated with accelerated bone loss and development of secondary osteoporosis, and consequently osteoporotic fractures *(Anwar MJ, et al., 2014; Lazzari AA, et al., 2013;*

Phabphal K, et al., 2013). Other unique risk factors, including the use of AEDs with sedative effect, have been described as playing important roles in increasing the risk of fractures in the epileptic population. A deficit of sun exposure, excessive alcohol and tobacco use, and poor dietary habits, are also considered to be responsible for the increased prevalence of osteoporosis in both the male and female epileptic population *(Lazzari AA, et al., 2013)*.

This thesis focuses on the newer antiepileptic drugs, in which the effect on bone metabolism is not fully known.

Levetiracetam (LEV), (*S*)-2-(2-Oxopyrrolidin-1-yl)butanamide, an analog of piracetam, is a relatively new broad-spectrum AED with a favourable tolerability and efficacy profile and a low potential for drug interactions. LEV is used in treating partial, generalized and myoclonic seizures *(Nissen-Meyer LS, et al., 2007).* Pharmacokinetic studies indicate fairly prompt and complete absorption and distribution. Elimination is renal. Interaction studies have shown no effect on the metabolism of other compounds, nor the converse *(Levy RH, et al., 2002)*. Despite the wide therapeutic use of LEV, to our knowledge there has been only one animal study, which reports changes in the biomechanical

strength properties of femoral bones in rats, along with documentation of changes in BMD and biochemical markers of bone turnover. This study demonstrates a biphasic dose-dependent effect of LEV on biomechanical bone strength, which may be related to microstructural changes in bone matrix *(Nissen-Meyer LS, et al., 2007).*

Lacosamide (LCM) (SPM 927, formerly harkeroside), the R-enantiomer of 2-acetamido-*N*-benzyl-3-methoxypropionamide, is a chemical compound with anticonvulsant and anti-nociceptive properties. LCM significantly reduces seizure frequency in adult patients with uncontrolled partial-onset seizures. The proposed primary mode of action includes selective enhancement of the slow inactivation of voltage-gated sodium channels (without affecting fast inactivation) *(Michelhaugh SK, et al., 2015).* In November 2007, a new drug application was filed with the FDA for use of LCM as adjunctive therapy in the treatment of partial-onset seizures in adults with epilepsy. LCM was approved in Europe on September 3, 2008 as adjunctive therapy in the treatment of partial-onset seizures, with or without secondary generalization, for patients with epilepsy of 16 years or older *(Johannessen LC et al 2009, Halford JJ end Lapointe M, 2009).* Sex hormone deficiency increases the risk of developing antiepileptic drug-induced osteopathy (AEDs-O) *(Carbone LD, et al., 2010).*

Topiramate (TPM), 2,3:4,5-bis-*O*-(1-methylethylidene)-β-D-fructopyranose sulfamate, is a carbonic anhydrase inhibitor commonly used in patients with focal epilepsy *(Giannopoulou EZ, et al., 2015).* Preliminary evidence suggests its efficacy for treating generalized seizures, and that it may have a broad spectrum of efficacy similar to that of lamotrigine. TPM has especially favourable pharmacokinetic characteristics. It is well absorbed and it is eliminated primarily by the kidneys. It has a plasma half-life of approximately 24 hours. *(Levy RH, et al., 2002)*.

Lamotrigine (LTG), 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine. Clinical trial experience suggests that LTG also has a broad spectrum of antiepileptic efficacy. In monotherapy studies enrolling patients with new-onset epilepsy of all types, LTG was found to be as effective as carbamazepine or phenytoin, and better tolerated. LTG has efficacy also in the treatment of absence and myoclonic seizures *(Levy RH, et al., 2002).* Lamotrigine is eliminated almost entirely by glucuronide conjugation *(Perucca E, 1999).* Its half-life is approximately 24 hours when used as monotherapy or together with

non-interacting drugs *(Levy RH, et al., 2002)*.

4. MAJOR DEPRESSIVE DISORDER (MDD)

MDD is a common psychiatric disorder. Numerous studies have found MDD to be associated with accelerated bone loss leading to the development of low bone mineral density (BMD) or osteoporosis, which is dependent on the duration of depression. Interestingly, various increased anti-inflammatory and pro-inflammatory cytokines have also been implicated in influencing osteoclastic bone resorption resulting in a decreased BMD. An increase in pro-inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) occurs in depressive disorders, resulting in increased bone resorption *(Malik P, et al., 2013).* Low vitamin D levels have also been found in depressive patients, which may also contribute to BMD reduction *(Eskandari F, et al., 2007).* Other possible pathways leading to low BMD in depressive patients are excessive smoking, secondary alcohol consumption, dietary deficiencies with low body mass index (BMI), and long-term treatment with antidepressants *(Malik P, et al., 2013).* The mechanism of action of antidepressants in the regulation of bone tissue is not fully understood. Recent studies have found that transporters of serotonin may play a role in bone metabolism and that medications which affect these transporter systems may also affect bone metabolism *(Rabenda V, et al., 2013).*

Antidepressants are some of the most commonly prescribed drugs *(Wu*

Q, et al., 2012). The link between depression, antidepressant use, and osteoporosis is becoming more widely understood, and there is mounting evidence for an effect of depression and antidepressants on fracture rates *(Rizzoli R, et al., 2012).* Selective serotonin reuptake inhibitors (SSRIs) are recommended for first-line pharmacological management of depression because they are considered safer and better tolerated than other types of antidepressants *(Wu Q, et al., 2012).* Tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) are two of the most widely prescribed classes of antidepressants. The mechanisms of action of all these agents involve some impact on the serotonin system, though the degree of inhibition of the serotonin transporter (5-HTT) system may differ between classes *(Bruyère O end Reginster JY, 2014).* Wu et al studied the effect of SSRIs and TCAs on the risk of fractures. Meta-analysis has shown a greater risk of osteoporotic fracture (72%) for the groups treated with TCA or SSRI than for the non-SSRI and non-TCA groups. However, the basic mechanism of the relationship between osteoporotic fractures and SSRI remains unclear *(Wu Q, et al., 2012; Wu Q, et al., 2013).*

Serotonin is well known as a regulator of mood. An increase in synaptic availability of serotonin is known to have an antidepressant effect, and is involved in all or part of the mechanism of action of some of the most widely used antidepressants. However, serotonin also plays an important role centrally in functions such as appetite, sleep, sexual activity, and temperature, and acts

peripherally in the cardiovascular and gastrointestinal systems. There is increasing evidence that serotonin may also be an important regulatory agent in bone metabolism, notably bone mass (*Rizzoli R, et al., 2012).* Serotonin is synthesized by two different genes at two different sites and plays antagonistic functions on bone mass accrual at these two sites. When produced peripherally, serotonin acts as a hormone to inhibit bone formation. In contrast, when produced in the brain, serotonin acts as a neurotransmitter to exert a positive and dominant effect on bone mass accrual by enhancing bone formation and limiting bone resorption *(Bruyère O end Reginster JY, 2014).* Based on these findings, treatment with antidepressants that increase levels of serotonin in the synapses, should lead to increments in bone mass *(Rizzoli R, et al., 2012).*

Mirtazapine (MIRTA), 2–methyl-1,2,3,4,10,14b-hexahydropyrazino[2,1-a]pyrido[2,3-c][2]benzazepine, is the only representative of the noradrenergic and specific serotonergic antidepressant class. It is a novel antidepressant which has a unique dual mode of action. Mirtazapine affects norepinephrine transmission via blockade of central α_2–adrenoceptors and is a potent serotonin 5-HT_2 and 5-HT_3 receptor antagonist, thereby increasing serotonergic stimulation via the 5-HT_1 receptor. It has no significant affinity for dopamine receptors, a low affinity for muscarinic cholinergic receptors and no effect on monoamine reuptake *(Alam A, et al., 2013).*

Venlafaxine (VENLA), (*RS*)-1-[2-dimethylamino-1-(4-methoxyphenyl)-ethyl]cyclohexanol, is a phenethylamine derivative widely prescribed for the treatment of depression, and its mechanism of action is based on the inhibition of the reuptake of serotonin and noradrenaline (SNRI). Venlafaxine's efficacy is comparable to that of tricyclic antidepressants; however, the SNRI has fewer adverse effects. As such, the use of venlafaxine has increased in recent years *(Ebrahimi F, et al., 2015).* Although its potency at the 5-HTT is less than that of other SSRIs, venlafaxine also inhibits the norepinephrine transporter; however, it is considered serotonin-selective because its potency at the 5-HTT is more than 100 times its potency at the norepinephrine transporter *(Shea ML, et al., 2013).*

Trazodone (TRA), 2-{3-[4-(3-chlorophenyl)piperazin-1-yl]propyl}[1,2,4]triazolo[4,3-a]pyridin-3(2H)-one, is a structurally unique bicyclic antidepressant effective in the treatment of depressive disorders, and which appears to be less toxic than other antidepressant drugs following an acute overdose. It inhibits the reuptake of serotonin (5-hydroxytryptamine), thereby

increasing serotonergic stimulation via the 5-HT_1 receptor. Prolonged-release trazodone is equally effective as some selective serotonin reuptake inhibitors, but has fewer adverse effects on sleep *(Zhang L, et al., 2014; Vanpee D, et al., 1999).*

5. AIMS OF THE STUDY

In our study we set out the following specific aims:

Specific aims:

1 a) To determine the effect of orchidectomy on bone metabolism in rats.

2 a) To determine the effect of selected antiepileptic drugs (levetiracetam, lacosamide, topiramate, lamotrigine) on bone metabolism in rats.

b) To determine the extent of the (negative) effect of the selected antiepileptic drugs in comparison to a control group.

3 a) To determine the effect of selected antidepressant drugs (mirtazapine, venlafaxine, trazodone) on bone metabolism in rats.

b) To determine the extent of the (negative) effect of the selected antidepressant drugs in comparison to a control group.

6. MATERIAL AND METHOD

All animals received humane care in accordance with the guidelines set by the institutional Animal Use and Care Committee of Charles University, Prague, Faculty of Medicine in Hradec Kralove, Czech Republic. The protocols of the experiment were approved by the same committee. The experiments used eight-week-old male albino Wistar rats (Biotest s.r.o., Konarovice, Czech Republic). The animals were hosted in groups of 4 in plastic cages. During the experimental period the animals were maintained under controlled conventional conditions (12 hours light and 12 hours dark, temperature 22±2^{o}C, air humidity 30–70 %). Tap water and standard laboratory diet (SLD, VELAS, a.s., Lysa nad Labem, Czech Republic) or SLD enriched with drugs were given *ad libitum*. The weights of the rats were monitored once a week.

6.1. Antiepileptics

- Levetiracetam (Levetiracetam, UCB Pharma)
- Lacosamide (Lacosamid, UCB Pharma)
- Lamotrigine (Lamotrigine, Glenmark)
- Topiramate (Topiramat, Glenmark)

6.2. Antidepressants

- Mirtazapine (Mirtazapin Krka, Czech republic)
- Trazadone (Trazodoni hydrochloridum, Medicom International s.r.o., Brno Czech republic)
- Venlafaxine (Venlafaxin TEVA RETARD, Teva Pharmaceuticals s.r.o, Czech republic)

6.3. Experiments

6.3.1. 1st Experiment

- Rats were fed with SLD enriched with the selected drugs during a 12 week period; n = 8.

1. CON-ORX: orchidectomised control fed with SLD
2. LEV-ORX: orchidectomised rat fed with SLD enriched with LEV (101 mg/25 g of the diet; Levetiracetam, UCB Pharma)
3. LCM–ORX: orchidectomised rat fed with SLD enriched with LCM (18 mg/25 g of the diet; Lacosamid, UCB Pharma)
4. LTG–ORX: orchidectomised rats fed with SLD enriched with LTG (39 mg/25 g of the diet; Lamotrigine, Glenmark)
5. TPM–ORX: orchidectomised rats fed with SLD enriched with TPM (23 mg/25 g of the diet; Topiramat, Glenmark)

6.3.2. 2nd Experiment

- Rats were fed with SLD enriched with the selected drugs during a 12 week period; n = 8.

  1. CON-ORX: orchidectomised control fed with SLD

  2. MIRTA-ORX: orchidectomised rat fed with SLD enriched with MIRTA (1,98 mg/25g of the diet; Mirtazapin Krka, Czech republic)

  3. VENLA–ORX: orchidectomised rat fed with SLD enriched with VENLA (12 mg/25g of the diet; venlafaxin TEVA RETARD, Teva Pharmaceuticals s.r.o, Czech republic)

  4. TRA–ORX: orchidectomised rats fed with SLD enriched with TRA (12 mg/25g of the diet; Trazodoni hydrochloridum, Medicom International s.r.o., Brno Czech republic)

6.4. Analysis

6.4.1. Bone homogenates

Bone homogenate was prepared from the tibiae. After animal sacrifice, both tibiae were carefully excised; after removal of all the surrounding skin, muscle and other soft tissue, they were stored at -80°C until required. The proximal part of the bone (0.1 g) was disrupted and homogenized in 1.5 ml of phosphate buffer (PBS, PAA Laboratories GmbH, Pasching, Austria) with a MagNA Lyser instrument (Roche Applied Science, Germany) at 6500 rpm for 20s, and cooled on the MagNA Lyser Cooling Block. This procedure was repeated three times. The raw tissue homogenate was centrifuged at 10,000 g at 4°C for 10 min, and the resulting supernatant was collected and stored at -80°C.

Levels of the markers carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), amino-terminal propeptide of procollagen type I (P1NP), bone alkaline phosphatase (BALP), osteoprotegerin (OPG), bone morphogenetic protein 2 (BMP-2) and sclerostin were determined in this bone homogenate, also using the ELISA method.

Levels of markers of bone turnover were determined using kits from the firm Uscn Life Science Inc., Wuhan, China (P1NP, Procollagen I N-Terminal Propeptide, pg/mL; OPG, Osteoprotegerin, pg/mL; IGF-1 Insulin Like Growth

Factor 1, pg/mL; CTX-I, Cross Linked C-Telopeptide Of Type I Collagen; pg/mL; BALP, bone alkaline Phosphatase, ng/ml; BMP-2, Bone Morphogenetic Protein 2, pg/mL; sclerostin, ng/mL).

6.4.2. Analysis of serum levels of drugs

- **Levetiracetam**

Concentrations of levetiracetam in the samples were determined by a modified high-performance liquid chromatography method with UV photodiode-array detection *(Lancelin F, et al., 2007).* After alkalinization of the sample (0.05 mL) levetiracetam and internal standard UCB 17025 were extracted into dichloromethane. Organic solvent was evaporated and the residue was dissolved and injected for HPLC analysis. Compounds were separated on a Zorbax SB-C8 column (Agilent Technologies, USA) at flow rate 1.1 mL/min. The mobile phase was composed of 10% acetonitrile, 7% methanol and 83% of a 20 mM phosphate buffer pH 6.7 with 0.1% triethylamine. UV detection was performed at a wavelength of 200 nm.

- **Lacosamide**

LCM was assayed by modified high-performance liquid chromatography with diode array detection *(Greenaway C, et al., 2010).* Sample preparation

included precipitation of plasma proteins: 200 µl of acetonitrile and 20 µl of zinc sulphate solution (10%) were added to 100 µl of plasma samples in 1.5-mL polypropylene centrifugation tubes. The tubes were vortexed for 120 seconds and centrifuged at 15,000 rpm for 10 minutes. The supernatant (30 µl) was injected into the HPLC system. Analysis was performed on a 2695 Waters Separations Module equipped with 996 photodiode array detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). Data acquisition and processing were provided with Empower Software (Waters). The analytical column was Zorbax SB-C8 (Agilent Technologies) – 150 x 4.6 mm, 3.5 µm. The analytical precolumn was Symmetry C18 Guard Column – 20 x 3.9 mm, 5 µm (Waters). The mobile phase was pumped at flow rate 0.8 ml/min and consisted of acetonitrile:formic acid 0.1 % (30:70, v/v). Temperature on the column was set at 30^{O}C, and injection volume was 30 µl. LCM concentration was determined at a wavelength of 215 nm *(Greenaway C, et al., 2010).*

- **Topiramate**

Determination of topiramate in the samples was performed using the gas chromatography-mass spectrometry method. This method was a modification of a bioanalytical method published previously *(Malakova J, et al., 2007).* The procedure included liquid-liquid extraction of 0.05 mL of the alkalinized sample with ethyl acetate. Trimethylanilinium hydroxide was used for flash methylation of topiramate and internal standard 5-(*p*-methylphenyl)-5-phenylhydantoin. Ions

of m/z 352 (for the topiramate derivative) and m/z 296 (for the internal standard derivative) were recorded for data evaluation.

- **Lamotrigine**

Concentrations of lamotrigine were measured using a modified method of high-performance liquid chromatography with UV photodiode-array detection *(Malakova J, et al., 2007).* Liquid-liquid extraction of a 0.05 mL alkalinized sample was carried out into ethyl acetate. After evaporation of the organic phase, the residue was dissolved in methanol. Lamotrigine and the internal standard BW 725C 78 were separated on a Symmetry C18 column (Waters, USA) 150 x 4.6 mm I.D., 5 µm particle size and Symmetry C18 Guard Column (20x3.9 mm I.D.). The mobile phase at isocratic flow rate of 1 mL/min contained acetonitrile (28%) and 6 mM phosphate buffer pH 6.8 (72%). The eluate was monitored at a wavelength of 306 nm.

- **Mirtazapine**

Serum levels of mirtazapine were determined using the HPLC-MS system. Sample preparation included precipitation of plasma proteins – 500 µl of 40 mM zinc sulfate in 66 % methanol was added to a polypropylene tube containing 500 µl of plasma sample and 50 µl of 2,000 ng/ml reserpine as internal standard (IS). Chromatographic separation was performed on a Hypersil GOLD column (Thermo scientific) 50 x 21 mm / 5 um with analytical precolumn (C18, 4 x 2.0

mm ID). Gradient elution using two solvents - 0.05 M formic acid (A) and acetonitrile (B) was started with 15 % solvent B that was increased to 65 % over 3 min and maintained for 2 minutes, and then was column equilibrated at 15% B for 2 min (total run time 7 min). The mobile phase flow was set to 0.2 mL/min and an aliquot of 10 µL was injected. LTQ XL (Thermo Fisher Scientific Corp.) was used as mass spectrometer with linear ion trap operating on electrospray ionization (ESI) at positive MS2 voltage 4.5 kV. Excalibur software was used for data analysis. For quantification a calibration curve was compiled relative to IS *(Borges NC, et al., 2012).*

- Venlafaxine

Serum levels of venlafaxine were determined using the HPLC-MS system. Sample preparation included precipitation of plasma proteins – 500 µl of 40 mM zinc sulfate in 66 % methanol was added to a polypropylene tube containing 500 µl of plasma sample and 50 µl of 2,000 ng/ml reserpine as internal standard (IS). Chromatographic separation was performed on a Hypersil GOLD column (Thermo scientific) 50 x 21 mm / 5 um with analytical precolumn (C18, 4 x 2.0 mm ID). Gradient elution using two solvents – 0.05 M formic acid (A) and acetonitrile (B) was started with 15 % solvent B that was increased to 65 % over 3 min and maintained for 2 minutes, and then was column equilibrated at 15% B for 2 min (total run time 7 min). The mobile phase flow was set to 0.2 mL/min and an aliquot of 10 µL was injected. LTQ XL (Thermo Fisher Scientific Corp.)

was used as mass spectrometer with linear ion trap operating on electrospray ionization (ESI) at positive MS2 voltage 4.5 kV. Excalibur software was used for data analysis. For quantification a calibration curve was compiled relative to IS *(Borges NC, et al., 2012).*

- Trazodone

Serum levels of trazodone were determined using the HPLC-MS system. Sample preparation included precipitation of plasma proteins – 500 µl of 40 mM zinc sulfate in 66 % methanol was added to a polypropylene tube containing 500 µl of plasma sample and 50 µl of 2,000 ng/ml reserpine as internal standard (IS). Chromatographic separation was performed on a Hypersil GOLD column (Thermo scientific) 50 x 21 mm / 5 um with analytical precolumn (C18, 4 x 2.0 mm ID). Gradient elution using two solvents - 0.05 M formic acid (A) and acetonitrile (B) was started with 15 % solvent B that was increased to 65 % over 3 min and maintained for 2 minutes, and then was column equilibrated at 15% B for 2 min (total run time 7 min). The mobile phase flow was set to 0.2 mL/min and an aliquot of 10 µL was injected. LTQ XL (Thermo Fisher Scientific Corp.) was used as mass spectrometer with linear ion trap operating on electrospray ionization (ESI) at positive MS2 voltage 4.5 kV. Excalibur software was used for data analysis. For quantification a calibration curve was compiled relative to IS *(Borges NC, et al., 2012).*

6.4.3. Dual energy X-ray absorptiometry analysis

The rat bone mineral density (BMD, g/cm^2) was measured by means of dual energy X-ray absorptiometry (DEXA) on a Hologic Delphi A device (Hologic, MA, USA) at the Osteocentre of the Faculty Hospital Hradec Kralove, Czech Republic. The rats were examined thus on the last day of experiment – before sacrifice. Before measurements, a tissue calibration scan was performed with the Hologic phantom for the small animal. Bone mineral densities of the whole body, in the area of the lumbar vertebrae, and in the area of the femur were evaluated by computer using the appropriate software program for small animals (DEXA; QDR-4500A Elite; Hologic, Waltham, MA, USA). All animals were scanned by the same operator.

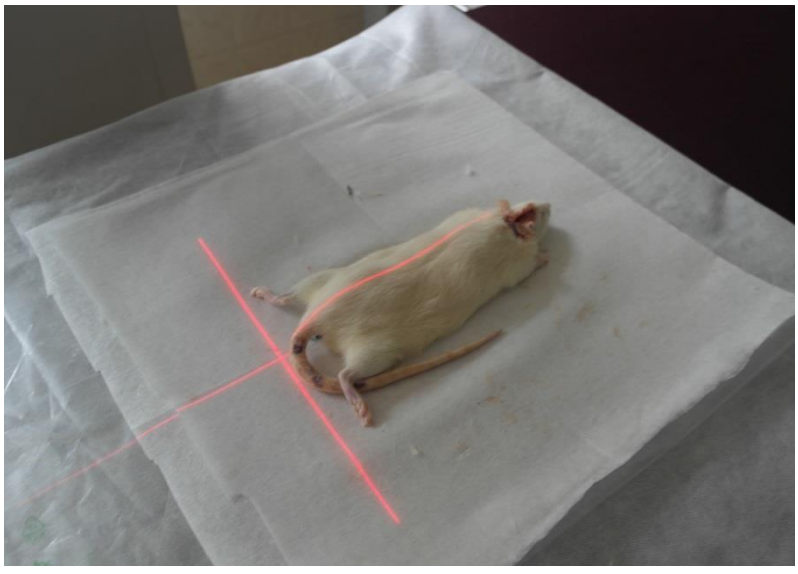

Figure 4. Position of rat during the BMD measurement by dual energy X-ray absorptiometry

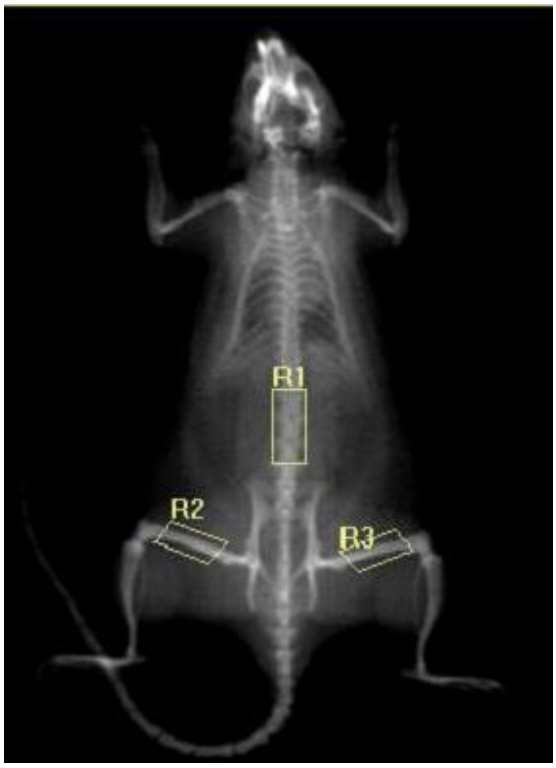

Figure 5. Evaluation of BMD in three areas of the rat skeleton

R1 – lumbar columna (L3-L5); R2 – left femur; R3 – right femur

6.4.4. Biomechanical testing procedure

Mechanical testing of the rat femoral shaft and femoral neck was done with a special custom-made electromechanical testing machine (Martin Kosek & Pavel Trnecka, Hradec Kralove, Czech Republic). For the three-point bending test, the femur was placed on a holding device with the two support points 18 mm apart. A small stabilizing preload to 10 N was applied in the anteroposterior direction to fix the bone between the contacts. A constant deformation rate of 6

mm/min was generated until maximal load failure, and the breaking strength (maximum load, N) was recorded. When the bone was broken, the thickness of the cortical part of the bone was measured by means of a sliding micrometer (OXFORD 0-25MM 30DEG POINTED MICROMETER, Victoria Works, Leicester, Great Britain). The proximal part of the femur was used for compression test of the femoral neck. The diaphysis of the bone was embedded into a container using a methacrylate resin, and a vertical load was applied to the top of the femoral head. A small stabilizing preload to 10 N was applied and increased at a constant speed of 6 mm/min until failure of the femoral neck. The breaking strength (maximum load, N) was recorded by the measuring unit (Digitalanzeiger 9180, Burster praezisiosmesstechnik gmbh & co kg, Gernsbach, Germany). All bones were analyzed by the same operator.

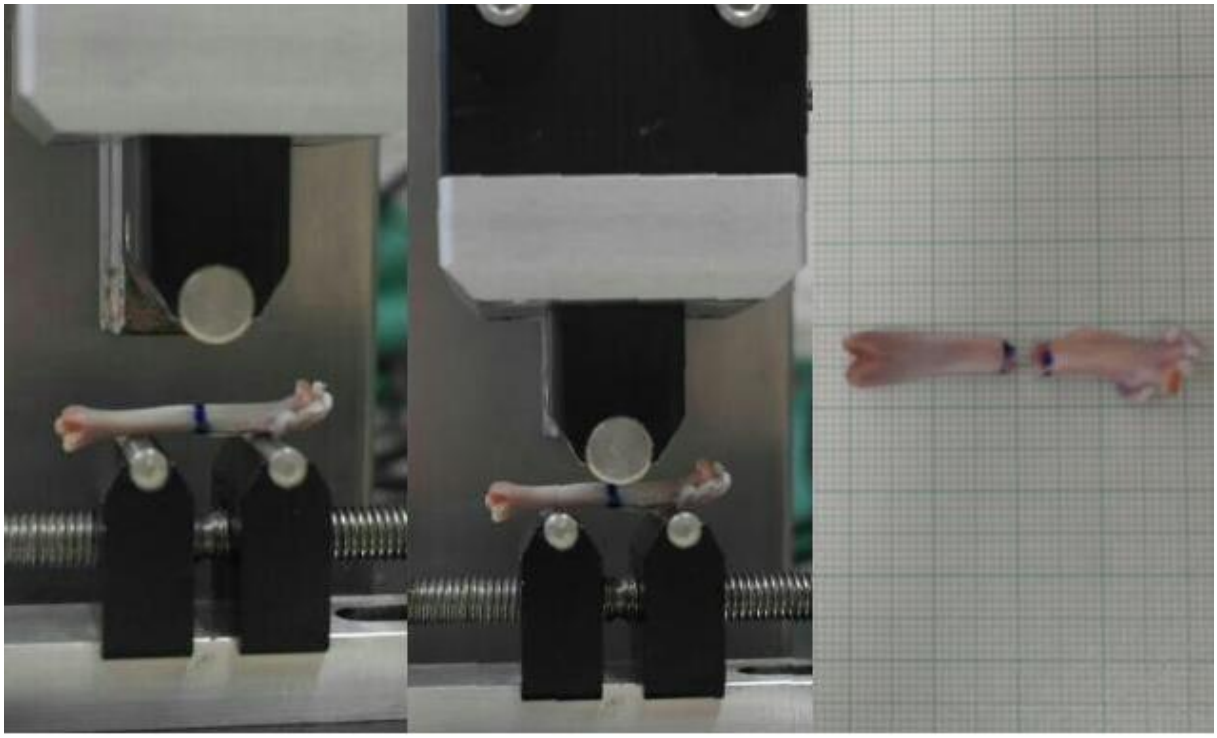

Figure 6. Biomechanical testing of the rat femoral shaft by three-point bending test in the anteroposterior direction

6.5. Statistical analysis

Statistical analysis was performed using the program NCSS 2007 (Number Cruncher Statistical System, Kaysville, Utah, USA). The results are of measurements made after 12 weeks of the experiment, and are presented as the median and the 25th and 75th percentiles. Comparison of the parameters under study employed an analysis of variance with post-hoc multiple

comparison by Fisher's LSD test and Kruskal-Wallis non-parametrical analysis of variance with post-hoc multiple comparison by Dunn's test (with Bonferroni´s modifications). Differences were considered significant at $p < 0,05$.

7. RESULTS

7.1. The effect of orchidectomy on rat bone

Weight and body composition

The performed orchidectomy caused a decrease in weight and lean body mass in ORX in comparison with the SHAM group (Table 1).

Levels of bone markers

Bone markers from specimens of the proximal tibia were measured to assess the effects of orchidectomy and treatment on bone formation. Determination of the levels of bone turnover markers (OPG) revealed their decrease in ORX versus SHAM. Levels of sclerostin were significantly increased (Table 2).

Dual Energy X-Ray Absorptiometry

In ORX, a significant decrease in the BMD of the whole body and also in the area of the lumbar vertebrae and both femurs was demonstrated compared with SHAM (Table 3).

Biomechanical Properties

The performed orchidectomy resulted in a decrease in the length of both

femurs, and in the maximal breaking load of both femurs and the neck of the femur (table 4).

Parameter	SHAM	ORX	P-value
Final body weight (g)	562 (541–622)	474 (460,25–490)*	**0,0018**
Fat (g)	90,5 (79,1 – 98,6)	103,85 (80 – 114,4)	0,2788
Lean body mass (g)	368,7 (359,85–399,35)	285,4 (278,6–303,3)*	**0,0003**

Tab. 1 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	SHAM	ORX	p-value
BALP (ng/ml)	20,09 (17,21 – 20,51)	18,31 (17,04 – 22,30)	0,7539
P1NP (pg/ml)	217,21 (164,71 – 248,82)	201,92 (182,58 - 235,28)	0,9015
OPG (pg/ml)	2,23 (2,12 – 2.46)	1,92 (1,56 – 2,08)*	**0,0111**
Sclerostin (ng/ml)	0.268 (0.221–0.279)	0,344 (0,288 – 0,381)*	**0,0085**
BMP (pg/ml)	25,14 (21,78 – 26,65)	24,88 (22,42 – 27,57)	0,7502
CTX	68,27 (48,72 – 78,41)	99,54 (79,09 – 110,30)*	**0,0365**

Tab. 2 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD (g/cm²)	SHAM	ORX	p-value
Whole body	0,184 (0,181 – 0,185)	0,164 (0,161 – 0,167)*	**0,00001**
Diaphysis right femur	0,199 (0,197 – 0,214)	0,181 (0,167 – 0,184)*	**0,00487**
Diaphysis left femur	0,194 (0,191 – 0,206)	0,176 (0,165 – 0,182)*	**0,00589**
Lumbar columna (L3-L5)	0,248 (0,234 – 0,251)	0,203 (0,193 – 0,213)*	**0,0002**

Tab. 3 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	SHAM	ORX	p-value
RF length (mm)	39,02 (38,44 – 39,25)	37,31 (36,58 – 37,96)*	**0,0027**
LF length (mm)	39 (38,27 – 39,24)	37,22 (36,88 – 37,67)*	**0,0007**
RF diameter (mm)	3,79 (3,72 – 3,9)	3,71 (3,58 – 3,88)	0,5028
LF diameter (mm)	3,77 (3,64 – 3,83)	3,65 (3,5 – 3,69)	0,3120
Cortical RF thickness	63 (61 - 65,5)	63 (61,5 – 65)	0,5920
Cortical LF thickness	64 (62 – 64)	63,5 (62,75 – 64,25)	1,0000
Maximal load of the right femoral shaft (N)	217,4 (198 – 225)	183 (179,25 – 191,25)*	**0,0299**
Maximal load of the left femoral shaft (N)	227 (224 – 236,5)	199 (191 – 217)*	**0,0269**
Maximal load of the right femoral neck (N)	160 (143 – 181,5)	152,43 (136 – 166)*	**0,0489**
Maximal load of the left femoral neck (N)	171 (139,5 – 184)	150.5 (130 – 165)*	**0,0484**

Tab. 4 Biomechanical and geometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

7.2. Antiepileptic drugs

The effect of levetiracetam on rat bone metabolism

Serum concentrations of drugs

The level of levetiracetam in the LEV-ORX group at the end of the experiment was 201,62 (191,9025 - 217,815) μmol/l, equivalent to therapeutic levels of the drug 35,2 - 235 μmol/l.

Weight and body composition

The weight of the experimental group decreased, but it is statistically insignificant versus the control group. DEXA revealed that the experimental group showed significantly decreased fat mass *versus* the control group (g). There were no significant differences in lean body mass between the experimental group and the control group (Table 5).

Levels of bone markers

As shown in Table 6, levetiracetam administration for 12 weeks caused a significant decrease in OPG and a borderline-significant increase in CTX-I. PINP and BALP were also increased but without statistical significance.

Dual Energy X-Ray Absorptiometry

Using densitometric measurements, we found loss of bone mineral density and bone mineral content of the right and left femur compared with control groups. There was no statistically significant difference in whole-body BMD between the study groups (Table 7).

Biomechanical Properties

The measured biomechanical and geometric parameters are shown in Table 8. There was no statistically significant difference in these parameters between rats receiving levetiracetam and control rats.

Parameter	LEV–ORX	CON-ORX	P-value
Final body weight	486,5 (472,75 - 490,5)	523 (489 – 543)	0,063
Fat (g)	71,35 (63,825 - 78,275)	90,3 (83,1 – 127,2)*	0,0087
Lean body mass (g)	335,4 (330,8 - 349,05)	326,2 (310,9 – 378,8)	0,9864

Tab. 5 Body weight and fat mass. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	LEV–ORX	CON-ORX	p-value
BALP (ng/ml)	1,490 (1,198 - 1,694)	1,161(0,794 - 1,382)	0,1806
P1NP (pg/ml)	87,395 (78,751 - 97,267)	78,977 (73,367 - 81,067)	0,1979
OPG (pg/ml)	49,628 (48,917 - 51,040)	54,671 (51,932 - 57,835)*	**0,0186**
IGF – I (pg/ml)	1,158 (1,117 - 1,192)	1,107 (1,081 - 1,167)	0,8158
BMP (pg/ml)	831,553 (704,383 - 1005,833)	846,841(633,905 - 985,989)	0,9467
CTX – I (pg/ml)	2,473 (1,365 - 3,149)	0,972 (0,802 - 1,537)	0,0661

Tab. 6 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD (g/cm^2)	LEV–ORX	CON-ORX	p-value
Whole body	0,176 (0,174 - 0,176)	0,175 (0,172 - 0,176)	0,5151
Diaphysis right femur	0,187 (0,177 – 0,1917)	0,195 (0,189 - 0,206)	0,1048
Diaphysis left femur	0,177 (0,173 – 0,189)	0,195 (0,194 – 0,199)*	**0,0181**
Lumbar columna (L3-L5)	0,212 (0,210 - 0,213)	0,207 (0,202 - 0,213)	0,3520

Tab. 7 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parameter	LEV–ORX	CON-ORX	p-value
RF length (mm)	37,13 (36,89 - 37,62)	37,4 (36,8 - 37,69)	0,9506
LF length (mm)	37,02 (36,845 - 37,075)	37,45 (36,72 - 37,955)	0,6601
RF diameter (mm)	3,69 (3,525 - 3,75)	3,63 (3,545 - 3,72)	0,7519
LF diameter (mm)	3,65 (3,335 - 3,72)	3,63 (3,515 - 3,945)	0,2092
Cortical RF thickness	0,7 (0,678 - 0,728)	0,705 (0,673 - 0,728)	0,5283
Cortical LF thickness	0,69 (0,658 - 0,74)	0,72 (0,673 - 0,735)	0,6069
Maximal load of the right femoral shaft (N)	207 (203,5 - 223,25)	216 (203,75 - 227,5)	0,5283
Maximal load of the left femoral shaft (N)	208 (195,25 - 223,5)	214,5 (196,75-233,75)	0,6332
Maximal load of the right femoral neck (N)	163,5 (151,75 - 171,25)	156 (136,5 - 171,25)	0,6546
Maximal load of the left femoral neck (N)	140,5 (134,25 - 148,25)	144 (135,5 – 152)	0,8945

Biomechanical and geometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

The effect of lacosamide on rat bone metabolism

Serum concentrations of drugs

The level of lacosamide in LCM-ORX group at the end of the experiment was 13.49 µmol/L (12.96 - 14.59), considerably below therapeutic levels of the drug 40 – 80 µmol/L

Weight and body composition

Comparison of body composition showed a significantly lower fat mass compared with the control group. The contrast in fat expressed as a percentage

was 18.3 % (100*(1-fatLCM/fatCON) between the groups - for details see Tab.9.

Levels of bone markers

Among the tested bone markers aminoterminal propeptide of procollagen type I was significantly lower in the LCM-ORX group. Particular values and a detailed list of the other followed parameters are displayed in Tab. 10.

Dual Energy X-Ray Absorptiometry

The mineral density of bone evaluated in the whole body and in the area of the lumbar vertebrae did not show any significant differences between the groups; however in the area of the left as well as the right femur we found significantly lower density in the LCM-ORX group compared to the control group (table 11).

Biomechanical Properties

The Mann-Whitney U test also showed that medians of biomechanical and geometric parameters of right and left femurs did not differ (Table 12).

Parameter	LCM-ORX	CON-ORX	p-value
Weight (g)	486 (471 – 499)	523 (489 – 543)	0,189
Fat (g)	73.8 (65,1 – 85,2)	90,3 (83,1 – 127,2)*	**0,024**
Lean body mass (g)	340,9 (326,9 – 350,9)	326,2 (310,9 – 378,8)	0,495

Tab. 9 Body weight and fat mas. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	LCM–ORX	CON-ORX	p-value
BALP (ng/ml)	1,490 (1,198 - 1,694)	1,161(0,794 - 1,382)	0,495
P1NP (pg/ml)	49,4 (34,5 – 67,6)	78,977 (73,367 - 81,067)*	**0,005**
OPG (pg/ml)	57,9 (51,1 – 54,5)	54,671 (51,932 - 57,835)	0,372
IGF – I (pg/ml)	1,17 (1,05 – 1,29)	1,107 (1,081 - 1,167)	0,564
BMP (pg/ml)	824 (763 – 1055)	846,841(633,905 - 985,989)	0,958
CTX – I (pg/ml)	0,90 (0,64 – 1,63)	0,972 (0,802 - 1,537)	0,772

Tab. 10 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD (g/cm^2)	LCM–ORX	CON-ORX	p-value
Whole body	0,175 (0,171 - 0,176)	0,175 (0,172 - 0,176)	0,066
Diaphysis right femur	0,175 (0,160 – 0,179)	0,195 (0,189 - 0,206)*	**0,001**
Diaphysis left femur	0,179 (0,169 – 0,185)	0,195 (0,194 – 0,199)*	**0,004**
Lumbar columna (L3-L5)	0,212 (0,207 - 0,223)	0,207 (0,202 - 0,213)	0,793

Tab. 11 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parameter	LCM-ORX	CON-ORX	p-value
RF length (mm)	37,4 (37,2 – 37,8)	37,4 (36,8 - 37,69)	0,958
LF length (mm)	37.3 (36,9 – 38,6)	37,45 (36,72 - 37,955)	0,495
RF diameter (mm)	3,65 (3,47 – 3,95)	3,63 (3,545 - 3,72)	0,753
LF diameter (mm)	3,64 (3,62 – 3,67)	3,63 (3,515 - 3,945)	0,958
Cortical RF thickness	0,695 (0,680 – 0,740)	0,705 (0,673 - 0,728)	0,958
Cortical LF thickness	0,705 (0,695 – 0,750)	0,72 (0,673 - 0,735)	0,263
Maximal load of the right femoral shaft (N)	224 (204 – 235)	216 (203,75 - 227,5)	0,564
Maximal load of the left femoral shaft (N)	218 (207 – 229)	214,5 (196,75-233,75)	1,000
Maximal load of the right femoral neck (N)	160 (138 – 178)	156 (136,5 - 171,25)	0,355
Maximal load of the left femoral neck (N)	146 (128 – 163)	144 (135,5 – 152)	0,897

Tab. 12 Biomechanical and geometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

The effect of lamotrigine on rat bone metabolism

Serum concentrations of drugs

The level of lamotrigine in the LTG-ORX group at the end of the experiment was 77.74 μmol/L (72.28 - 84.22); therapeutic levels of the drug are 12-66 μmol/L.

Weight and body composition

The weight of the experimental group decreased in a statistically significant manner. There was also a significant decrease in fat mass and lean body mass compared with the control group (Table 13).

Levels of bone markers

As shown in table 14, there were no statistical changes in the levels of bone markers. Only the levels of BALP were decreased in the LTG-ORX group compared control group, but not statistically significant.

Dual Energy X-Ray Absorptiometry

Using densitometric measurements, we found loss of bone mineral density of the whole body, right and left femur compared with the control groups (Table 15).

Biomechanical Properties

Testing of the mechanical strength of the bone tissue by means of three-point bending revealed a statistically significant decrease in maximal load values versus the control group (Table 16).

Parameter	LTG-ORX	CON-ORX	p-value
Weight (g)	415,5 (402,25 – 449,25)	523 (489 – 543)*	**0,0014**
Fat (g)	64,4 (59,9 – 70,775)	90,3 (83,1 – 127,2)*	**0,0026**
Lean body mass (g)	295,15 (281,85 – 310,8)	326,2 (310,9 – 378,8)*	**0,0432**

Tab. 13 Body weight and fat mass. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	LTG-ORX	CON-ORX	p-value
BALP (ng/ml)	0,868 (0,838 - 0,963)	1,161(0,794 - 1,382)	0,1214
P1NP (pg/ml)	86,84 (80,244 – 97,172)	78,977 (73,367 - 81,067)	0,1444
OPG (pg/ml)	52,224 (51,102 – 54,545)	54,671 (51,932 - 57,835)	0,4309
IGF – I (pg/ml)	1,269 (1,243 – 1,295)	1,107 (1,081 - 1,167)	0,0734
BMP (pg/ml)	817,627 (774,578 – 890,548)	846,841(633,905 - 985,989)	0,7541
CTX – I (pg/ml)	0,568 (0,53 – 0,675)	0,972 (0,802 - 1,537)	0,1288

Tab. 14 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD (g/cm^2)	LTG-ORX	CON-ORX	p-value
Whole body	0,168 (0,160 - 0,172)	0,175 (0,172 - 0,176)*	**0,0026**
Diaphysis right femur	0,169 (0,163 – 0,177)	0,198 (0,188 - 0,211)	0,1033
Diaphysis left femur	0,174 (0,1666 – 0,179)	0,197 (0,192 – 0,199)*	**0,0039**
Lumbar columna (L3-L5)	0,207 (0,201 - 0,210)	0,214 (0,210 - 0,223)	0,6899

Tab. 15 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parameter	LTG-ORX	CON-ORX	p-value
RF length (mm)	37,05 (36,485 – 37,56)	37,4 (36,8 - 37,69)	0,5660
LF length (mm)	36,78 (36,12 - 37,11)	37,45 (36,72 - 37,955)	0,2492
RF diameter (mm)	3,5 (3,435 – 3,565)	3,63 (3,545 - 3,72)	0,0502
LF diameter (mm)	3,51 (3,47 – 3,54)	3,63 (3,515 - 3,945)	0,2827
Cortical RF thickness (mm)	0,69 (0,65 – 0,713)	0,705 (0,673 - 0,728)	0,4700
Cortical LF thickness (mm)	0,67 (0,66 – 0,68)	0,72 (0,673 - 0,735)	0,2099
Maximal load of the RF femoral shaft (N)	186 (166,75 – 192)	216 (203,75 - 227,5)*	**0,0014**
Maximal load of the LF femoral shaft (N)	180 (169,75 – 198)	214,5 (196,75-233,75)*	**0,0103**
Maximal load of the right femoral neck (N)	119 (110 -123)	156 (136,5 - 171,25)*	**0,0003**
Maximal load of the left femoral neck (N)	117 (109 -120)	144 (135,5 – 152)*	**0,0013**

Tab. 16 Biomechanical and geometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

The effect of topiramate on rat bone metabolism

Serum concentrations of drugs

The level of topiramate in the TPM-ORX group at the end of the experiment was 22.04 μmol/L (21.64-22.71), equivalent to therapeutic levels of the drug 15-75 μmol/L.

Weight and body composition

The weight of the experimental group decreased in a statistically significant manner. There was also a significant decrease in fat mass compared with the control group (Table 17).

Levels of bone markers

No statistically significant difference in bone turnover markers was found (Table 18).

Dual Energy X-Ray Absorptiometry

A significant loss of BMD was found for the whole body and the right and left femurs for both experimental groups compared with the control group (table 19).

Biomechanical Properties

The Mann-Whitney U test also showed that medians of biomechanical and geometric parameters of right and left femurs did not differ (Table 20).

Parameter	TPM-ORX	CON-ORX	p-value
Final body weight (g)	486 (474,75 – 493)	523 (489 – 543)	0,0495
Fat (g)	73,6 (67 – 83)	90,3 (83,1 – 127,2)*	**0,0202**
Lean body mass (g)	334 (321,25 – 344,35)	326,2 (310,9 – 378,8)	0,8525

Tab. 17 Body weight and fat mass. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	TPM-ORX	CON-ORX	p-value
BALP (ng/ml)	1,528 (1,378 - 1,664)	1,161(0,794 - 1,382)	0,1046
P1NP (pg/ml)	87,401 (77,619 – 94,04)	78,977 (73,367 - 81,067)	0,1540
OPG (pg/ml)	52,613 (50,628 – 53,656)	54,671 (51,932 - 57,835)	0,4001
IGF – I (pg/ml)	1,111 (1,092 – 1,197)	1,107 (1,081 - 1,167)	0,0733
BMP (pg/ml)	834,857 (707,31 – 1057,472)	846,841(633,91 - 986)	0,9753
CTX – I (pg/ml)	0,505 (0,478 – 0,652)	0,972 (0,802 - 1,537)	0,0933

Tab. 18 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD (g/cm^2)	TPM-ORX	CON-ORX	p-value
Whole body	0,168 (0,166 – 0,169)	0,175 (0,172 - 0,176)*	**0,0406**
Diaphysis right femur	0,179 (0,176 – 0,188)	0,195 (0,189 - 0,206)*	**0,0053**
Diaphysis left femur	0,179 (0,173 – 0,190)	0,195 (0,194 – 0,199)*	**0,0136**
Lumbar columna (L3-L5)	0,196 (0,191 – 0,207)	0,207 (0,202 - 0,213)	0,3934

Tab. 19 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parameter	TPM-ORX	CON-ORX	p-value
RF length (mm)	37,26 (37,22 – 37,37)	37,4 (36,8 - 37,69)	0,9778
LF length (mm)	37,3 (37,22 – 37,41)	37,45 (36,72 - 37,955)	0,9017
RF diameter (mm)	3,52 (3,48 – 3,62)	3,63 (3,545 - 3,72)	0,1544
LF diameter (mm)	3,57 (3,5 – 3,64)	3,63 (3,515 - 3,945)	0,1010
Cortical RF thickness	0,74 (0,698 – 0,753)	0,705 (0,673 - 0,728)	0,3400
Cortical LF thickness	0,75 (0,693 – 0,75)	0,72 (0,673 - 0,735)	0,5346
Maximal load of the right femoral shaft (N)	194,5 (188,75 – 201,5)	216 (203,75 - 227,5)	0,0839
Maximal load of the left femoral shaft (N)	197,5 (192 – 207,75)	214,5 (196,75-233,75)	0,1261
Maximal load of the right femoral neck (N)	134,5 (126,5 – 149,75)	156 (136,5 - 171,25)	0,1804
Maximal load of the left femoral neck (N)	139 (121,5 – 153,5)	144 (135,5 – 152)	0,4415

Tab. 20 Biomechanical and geometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parametr	LEV-ORX	LCM-ORX	TPM-ORX	LTG-ORX	CON-ORX
P1NP (pg/ml)	87,40 (78,75 - 97,267)	49,4 (34,5 – 67,6)	87,40 (77,62 – 94,04)	86,84 (80,24 – 97,17)	78,98 (73,37 - 81,07)
BALP (ng/ml)	1,49 (1,20 - 1,69)*	1,49 (1,20 - 1,69)	1,528 (1,378 - 1,664)	0,868 (0,838 - 0,963)	1,161(0,794 - 1,382)
OPG (pg/ml)	49,63 (48,92 - 51,04)	57,9 (51,1 – 54,5)**	52,61 (50,63 – 53,66)	52,22 (51,10 – 54,55)	54,67 (51,93 - 57,84)
CTX (pg/ml)	2,47 (1,37 - 3,15)	0,90 (0,64 – 1,63)	0,505 (0,478 – 0,652)	0,568 (0,53 – 0,675)	0,972 (0,802 - 1,537)
BMD whole body (g/cm^2)	0,176 (0,174 - 0,176)	0,175 (0,171 – 0,176)	0.168 (0.166 – 0.169)	0,168 (0,16 – 0,172)*	0,175 (0,172 - 0,176)
BMD RF (g/cm^2)	0,187 (0,177 – 0,1917)	0,175 (0,160 – 0,179)***	0.179 (0.176 – 0.188)*	0,169 (0,163 – 0,177)***	0,195 (0,189 - 0,206)
BMD LF (g/cm^2)	0,177 (0,173 – 0,189)	0,179 (0,169 – 0,185)***	0.179 (0.173 – 0.190)*	0,174 (0,167 – 0,179)***	0,195 (0,194 – 0,199)
Maximal load of the right femoral shaft	207 (204 - 223)	224 (204 – 235)	195 (189 – 202)	186 (167 – 192)**	216 (204 - 228)
Maximal load of the left femoral shaft (N)	208 (195 - 224)	218 (207 – 229)	198 (192 – 208)	180 (170 – 198)*	215 (197 - 234)
Maximal load of the right femoral neck	164 (152 - 171)	160 (138 – 178)	135 (127 – 150)	119 (110 -123)**	156 (137 - 171)
Maximal load of the left femoral neck (N)	141 (134 - 148)	146 (128 – 163)	139 (122 – 154)	117 (109 -120)*	144 (136 – 152)

Tab. 21 Comparison of chosen parameters in all selected antiepileptic drugs to the control group; *p < 0.05; **p < 0,01, ***p < 0,001.

7.3. Antidepressant drugs

The effect of mirtazapine on rat bone metabolism

Serum concentrations of drugs

The level of mirtazapine in the MIRTA-ORX group at the end of the experiment was 0,060 mg/L (0,045–0,060 mg/L), equivalent to therapeutic levels of the drug 0,030 – 0,080 mg/L.

Weight and body composition

The weight of the experimental MIRTA-ORX group decreased, but this was statistically non-significant versus the control group. DXA revealed that the experimental group showed an increase in fat mass versus the control group. There were no significant differences in lean body mass between the experimental and the control group (Table 22).

Levels of bone markers

In the ORX control group, the levels of OPG, BALP, P1NP and BMP-2 were decreased versus the MIRTA-ORX group, but those of P1NP and BMP-2 not significantly so. The results from ELISA are shown in Table 23.

Dual Energy X-Ray Absorptiometry

In the MIRTA-ORX group there was a significant decrease in BMD of the whole body and both femurs, but the BMD of the lumbar vertebrae was unchanged versus the ORX control group. The results are shown in Table 24.

Biomechanical Properties

After mirtazapine administration there was a statistically significant decrease in length, and a decrease in thickness (not statistically significant) of the cortical bone as compared with the ORX group. There was a statistically significant decrease in maximal load of the femoral neck, but the maximal load of the femoral shaft was unchanged versus the ORX group (Table 25).

Parameter	MIRTA-ORX	CON-ORX	p-value
Final body weight (g)	452 (431,25 – 486,25	474 (460,25 – 490)	0,2337
Fat (g)	99,75 (92,111,03)	103,85 (80 – 114,4)	0,7265
Lean body mass (g)	265,95 (261,48–289,78)	285,4 (278,6 – 303,3)	0,2827

Tab. 22 Body weight and fat mass. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	MIRTA-ORX	CON-ORX	p-value
BALP (ng/ml)	18,31 (17,04 - 22,30)	20,09 (17,21 – 20,51)	0,7539
P1NP (pg/ml)	201,92 (182,58 – 235,28)	217,21 (164,71 - 248,82)	0,9015
OPG (pg/ml)	1,44 (1,32 – 1,55)	1,92 (1,56 – 2,08)*	**0,0181**
Sclerostin (ng/ml)	0,344 (0,288 – 0,381)	0,268 (0,221 – 0,279)*	**0,0085**
BMP (pg/ml)	24,88 (22,42 – 27,57)	25,14(21,78 – 26,65)	0,7502
CTX-I (pg/ml)	58,79 (55,62 – 60,64)	68,27 (48,72 - 78,41)	0,2637

Tab. 23 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD (g/cm²)	MIRTA-ORX	CON-ORX	p-value
Whole body	0.159 (0.158–0.166)	0,164 (0,161 - 0,167)	0.9872
Diaphysis right femur	0.171 (0.159–0.175)	0,181 (0,167 - 0,184)*	**0.0394**
Diaphysis left femur	0.161 (0.158–0.168)	0,176 (0,164 – 0,182)	0.0637
Lumbar columna (L3-L5)	0.200 (0.195–0.208)	0,203 (0,193 - 0,213)	0.8130

Tab. 24 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parameter	MIRTA-ORX	CON-ORX	p-value
RF length (mm)	36,44 (35,43 – 37,26)	37,31 (36,58 – 37,96)*	**0,0211**
LF length (mm)	36,08 (35,29 – 36,72)	37,22 (36,88 – 37,67)*	**0,0218**
RF diameter (mm)	3,65 (3,52 – 3,82)	3,71 (3,58 – 3,88)	0,4056
LF diameter (mm)	3,51 (3,37 – 3,67)	3,65 (3,5 – 3,69)	0,1989
Cortical RF thickness	60,5 (58,75 - 62)	63 (61,5 - 65)	0,0912
Cortical LF thickness	61,5 (58,5 – 63,25)	63,25 (62,75 – 64,25)	0,6601
Maximal load of the right femoral shaft (N)	196 (188 – 203,5)	183 (179,25 – 191,25)	0,1391
Maximal load of the left femoral shaft (N)	201,5 (184,5 – 220,5)	199 (191 – 217)	0,8903
Maximal load of the right femoral neck (N)	132 (123,7 – 137,5)	152,43 (136 – 166)*	**0,0486**
Maximal load of the left femoral neck (N)	133 (126,5 – 146,5)	150,5 (130 – 165)*	**0,0494**

Tab. 25 Biomechanical and geometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

The effect of venlafaxine on rat bone metabolism

Serum concentrations of drugs

The level of venlafaxine in the VEN-ORX group at the end of the experiment was 0,46 mg/L (0,3 – 0,625 mg/L), equivalent to therapeutic levels of the drug 0,2 – 0,75 mg/L.

Weight and body composition

DXA revealed that the experimental group showed a decrease in fat mass versus the control group. There were no significant differences in lean body mass between the experimental group and the control group (Table 26).

Levels of bone markers

The levels of BALP were decreased, while the levels of CTX-I and sclerostin were increased. The results from the ELISA determination are shown in Table 27.

Dual Energy X-Ray Absorptiometry

In the group VENLA-ORX there was a significant decrease in the BMD and BMC of both femurs. The results are shown in Table 28.

Biomechanical Properties

There were no significant differences in biomechanical testing measurement between the experimental group and the control group. The results are shown in Table 29.

Parameter	VENLA-ORX	CON-ORX	p-value
Final body weight (g)	434,5 (428 – 447)	474 (460,25 – 490)*	**0,0415**
Fat (g)	78,7 (70,65 - 85,4)	103,85 (80 – 114,4)*	**0,0217**
Lean body mass (g)	294,1 (288,9 – 309,8)	285,4 (278,6 – 303,3)	0,6601

Tab. 26 Body weight and fat mass. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	VENLA-ORX	CON-ORX	p-value
BALP (ng/ml)	7,15 (5,60 – 8,87)	20,09 (17,21 – 20,51)*	**0,0000**
P1NP (pg/ml)	210,21 (175,53 – 261,09)	217,21 (164,71 - 248,82)	0,7392
OPG (pg/ml)	1,64 (1,53 – 1,85)	1,92 (1,56 – 2,08)	0,3184
Sclerostin (ng/ml)	0,457 (0,405 – 0,484)	0,268 (0,221 – 0,279)*	**0,0000**
BMP (pg/ml)	22,22 (19,89 – 23,78)	25,14(21,78 – 26,65)	0,270
CTX-I (pg/ml)	99,54 (79,09 – 110,30)	68,27 (48,72 - 78,41)*	**0,0085**

Tab. 27 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD (g/cm²)	VENLA-ORX	CON-ORX	p-value
Whole body	0,158 (0156 – 0,165)	0,164 (0,161 - 0,167)	0,2688
Diaphysis right femur	0,161 (0,158 – 0,166)	0,181 (0,167 - 0,184)*	**0,010**
Diaphysis left femur	0,154 (0,151 – 0,157)	0,176 (0,164 – 0,182)*	**0,0037**
Lumbar columna (L3-L5)	0,202 (0,198 – 0,211)	0,203 (0,193 - 0,213)	0,8904

Tab. 28 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n =8

Parameter	VENLA-ORX	CON-ORX	p-value
RF length (mm)	37,08 (36,85 – 37,61)	37,31 (36,58 – 37,96)	0,5766
LF length (mm)	37,24 (36,88 – 37,83)	37,22 (36,88 – 37,67)	0,9236
RF diameter (mm)	3,54 (3,50 – 3,62)	3,71 (3,58 – 3,88)	0,1083
LF diameter (mm)	3,51 (3,39 – 3,54)	3,65 (3,5 – 3,69)	0,0723
Cortical RF thickness	65 (63,5 – 66,75)	63 (61,5 – 65)	0,3277
Cortical LF thickness	65 (61,5 – 67)	63,5 (62,75 – 64,25)	0,4831
Maximal load of the right	191 (183,75 – 213,5	183 (179,25 – 191,25)	0,1444
Maximal load of the left	190,5 (177,25 – 222,5)	199 (191- 217)	0,9283
Maximal load of the right	124,5 (121,75 – 133,25)	152,43 (136- 166)	0,1281
Maximal load of the left	124 (111 – 133,5)	150,5 (130 – 165)	0,0887

Tab. 29 Biomechanical and geometric parametrs. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

The effect of trazodone on rat bone metabolism

Serum concentrations of drugs

At the end of experiment the level of trazodone in the TRA-ORX group was 0,6 (0,5 – 0,625 mg/L), equivalent to therapeutic levels of the drug 0,5 – 2,5 mg/L.

Weight and body composition

DEXA revealed that the experimental group showed a decrease in fat mass versus the control group. There were no significant differences in lean body mass between the experimental group and the control group (Table 30).

Levels of bone markers

The levels of OPG and BALP were decreased. The levels of sclerostin were increased. The results from the ELISA determination are shown in table 31.

Dual Energy X-Ray Absorptiometry

There were no significant differences in BMD and BMC between the experimental group and the control group. The results are shown in Table 32.

Biomechanical Properties

There were no significant differences in biomechanical testing measurement between the experimental group and the control group. The results are shown in Table 33.

Parameter	TRA-ORX	CON-ORX	p-value
Final body weight (g)	528 (492,75 – 558,25)	474 (460,25 – 490)	0,1085
Fat (g)	65,7 (62,68 – 70)	103,85 (80 – 114,4)*	**0,0029**
Lean body mass (g)	311,5 (305 – 323,5)	285,4 (278,6 – 303,3)	0,0870

Tab. 30 Body weight and fat mass. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

Parameter	TRA-ORX	CON-ORX	p-value
BALP (ng/ml)	5,81 (5,64 – 6,46)	20,09 (17,21 – 20,51)*	**0,0028**
P1NP (pg/ml)	224,49 (200,89 – 248,29)	217,21 (164,71 - 248,82)	0,4487
OPG (pg/ml)	1,35 (1,26 – 1,41)	1,92 (1,56 – 2,08)*	**0,0014**
Sclerostin (ng/ml)	0,416 (0,354 – 0,460)	0,268 (0,221 – 0,279)*	**0,0095**
BMP (pg/ml)	2128 (19,81 – 23,39)	25,14(21,78 – 26,65)	0,1652
CTX-I (pg/ml)	61,37 (54,88 – 70,82)	68,27 (48,72 - 78,41)	0,4302

Tab. 31 Levels of bone markers. Data are expressed as median (25 th – 75 th percentiles), n=8; *p < 0.05.

BMD(g/cm²)	TRA-ORX	CON-ORX	p-value
Whole body	0,164 (0,160 – 0,166)	0,164 (0,161 - 0,167)	0,8016
Diaphysis right femur	0,174 (0,170 – 0,177)	0,181 (0,167 - 0,184)	0,7774
Diaphysis left femur	0,166 (0,157 – 0,169)	0,176 (0,164 – 0,182)	0,1715
Lumbar columna (L3-L5)	0,207 (0,202 – 0,210)	0,203 (0,193 - 0,213)	0,7589

Tab. 32 Densitometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parameter	TRA-ORX	CON-ORX	p-value
RF length (mm)	37,23 (36,69 – 37,66)	37,31 (36,58 – 37,96)	0,6571
LF length (mm)	37,73 (36,83 – 37,83)	37,22 (36,88 – 37,67)	0,6598
RF diameter (mm)	3,62 (3,53 – 3,67)	3,71 (3,58 – 3,88)	0,2891
LF diameter (mm)	3,7 (3,54 – 3,76)	3,65 (3,5 – 3,69)	0,7910
Cortical RF thickness	62,5 (59,75 – 64,5)	63 (61,5 – 65)	0,8106
Cortical LF thickness	63,5 (62,75 – 64,25)	64 (62 – 64)	0,6601
Maximal load of the right femoral shaft (N)	183 (179,25 – 191,25)	204 (196 – 208)	0,1234
Maximal load of the left femoral shaft (N)	206,5 (203 – 214,25)	199 (191- 217)	0,4726
Maximal load of the right femoral neck (N)	124 (113,5 – 144,5)	152,43 (136- 166)	0,2796
Maximal load of the left femoral neck (N)	141(128,75 – 154)	150,5 (130 – 165)	0,3937

Tab. 33 Biomechanical and geometric parameters. Data are expressed as median (25 th – 75 th percentiles), n = 8; *p < 0.05.

Parameter	MIRTA-ORX ORX	VENLA-ORX	TRA-ORX	CON-ORX
P1NP (pg/ml)	201,92 (182,58 – 235,28)	210,21 (175,53 – 261,09)	224,49 (200,89 – 248,29)	217,21 (164,71 - 248,82)
BALP (ng/ml)	18,31 (17,04 - 22,30)*	7,15 (5,60 – 8,87)***	5,81 (5,64 – 6,46)***	20,09 (17,21 – 20,51)
OPG (pg/ml)	1,44 (1,32 – 1,55)*	1,64 (1,53 – 1,85)	1,35 (1,26 – 1,41)**	1,92 (1,56 – 2,08)
Sclerostin (ng/ml)	0,344 (0,288 – 0,381)	0,457 (0,405 – 0,484)	0,416 (0,354 – 0,460)	0,268 (0,221 – 0,279)
CTX-I (pg/ml)	58,79 (55,62 – 60,64)	99,54 (79,09 – 110,30)	61,37 (54,88 – 70,82)	68,27 (48,72 - 78,41)
BMD whole body (g/cm^2)	0.159 (0.158–0.166)	0,158 (0156 – 0,165)	0,164 (0,160 – 0,166)	0,164 (0,161 – 0,167)
BMD RF (g/cm^2)	0.171 (0.159–0.175)	0,161 (0,158 – 0,166)	0,174 (0,170 – 0,177)	0,181 (0,167 – 0,184)
BMD LF (g/cm^2)	0.161 (0.158–0.168)	0,154 (0,151 – 0,157)	0,166 (0,157 – 0,169)	0,176 (0,165 – 0,185)
Maximal load of the right femoral shaft (N)	196 (188–203.5)	191 (183,75 – 213,5)	183 (179,25 – 191,25)	204 (196 – 208)
Maximal load of the left femoral shaft (N)	201.5 (184.5–220.5)	190,5 (177,25 – 222,5)	206,5 (203 – 214,25)	199 (191- 217)
Maximal load of the right femoral neck (N)	132 (123.7–137.5)*	124,5 (121,75 – 133,25)	124 (113,5 – 144,5)	152,43 (136- 166)
Maximal load of the left femoral neck (N)	133 (126.5–146.5)*	124 (111 – 133,5)	141(128,75 – 154)	150,5 (130 – 165)

Tab. 34 Comparison of chosen parameters in all selected antidepressant drugs to the control group

*p < 0.05; **p < 0,01, ***p < 0,001

8. DISCUSSION

The increase in life expectancy of the world population is associated with challenges regarding health issues. For instance, osteoporosis is a medical condition mostly observed in elderly people, in which the quality and quantity of the bone are severely affected. Not only for women but also for men, osteoporosis is recognized as an important public health issue *(Alghamdi HS, et al., 2014).*). Osteoporotic fractures are a significant cause of morbidity and mortality *(Wheater G, et al., 2013).*

8.1. The effect of orchidectomy on rat bone mass, structure and metabolism

Research on osteoporosis has so far been primarily performed on postmenopausal women, due to the high incidence of disease in this population. However, it is important to acknowledge that also men who had undergone spinal surgery had osteoporosis, which can result in higher reported morbidity. For this reason, research on male osteoporosis is essential *(Ryu SJ, et al., 2015).* We found in rats 12 weeks post-orchidectomy a significant decrease in body weight, bone mineral density and lean body mass, and an increase in fat mass. These results confirm the findings from previous studies in rats, that deficiency of androgens

negatively affects body composition, and establishes these animals as suitable models for investigating androgenic modulation of body composition *(Vanderschuerenet D, et al., 2000; Gentile MA, et al., 2010).*

8.2. The effect of the selected newer antiepileptic drugs on bone metabolism

Since the end of the 1960s, the effect of antiepileptic agents on bone has been examined in a number of papers. However, in the case of the novel antiepileptics, data are scarce: there are no prospective studies with a sufficiently long period of monitoring. The exact mechanism of action of selected antiepileptics (LEV, LCM, LTG, TPM) is yet to be defined. There are several anticipated mechanisms of action: changes in γ-aminobutyric acid (GABA) metabolism and turnover; inhibition of the excitatatory system (mainly glutamate); inhibition of neurotransmission by modulation of voltage-controlled sodium channels; and inhibition of T-type calcium channels *(Walker MC and Sander JW, 1999; Dooley M and Plosker GL, 2000; Lang DG, et al., 1993).*

The effect of levetiracetam on rat bone mass

We found that long-term LEV treatment significantly reduced BMD of the left femoral diaphysis. We observed a significant fall in OPG, and an increase in CTX-I of borderline statistical significance. No significant differences in biomechanical or geometric parameters of rat right and left femurs were observed. Adipose tissue significantly decreased in both absolute and relative terms, while body weight decreased at borderline statistical significance.

There are only a few and conflicting data in the literature concerning the effect of LEV on BMD. Nissen-Meyer and co-workers have reported in rats that LEV did not alter bone mass as judged by unaltered BMC and BMD. That paper suggests a dose-dependent (biphasic) effect of LEV on bone. Only low dose LEV (serum concentration 122 ± 41 umol/l) was associated with reduced biomechanical strength and reduced levels of serum osteocalcin, a marker of bone formation, but not high dose (serum LEV concentration 277 ± 65 umol/l). Their results suggest LEV may have a harmful effect on trabecular bone rather than cortical bone, which would be manifested in a change in BMD. Compared to this previous study *(Nissen-Meyer LS, et al., 2007),* our results suggest that in the ORX-rat model LEV may affect bone mass of the femoral diaphysis (cortical bone). The serum LEV concentration in our study (201.62 [191.9025–217.815] umol/l) was comparable to that reported in the high-dose group in the previous study *(Nissen-Meyer LS, et al., 2007).* However, because LEV was administered in different ways (SLD enriched with LEV *ad libitum* in the present study *versus* twice-daily gastric feeding in the Nissen-Meyer

paper), and since we don't know the timing of blood sampling relative to drug administration in Nissen-Meyer's paper, there is limited validity in comparing plasma concentrations of LEV between the two studies. Our results (in regard to OPG and CTX-I levels) suggest that LEV may suppress bone turnover. We may assume that the reduction in serum OPG levels could be explained by the inhibition of osteoblast activity.

In research on humans, there have been conflicting reports on bone strength and metabolism. One retrospective cross-sectional study suggested that LEV is more often associated with decreased BMD compared to the other AEDs (carbamazepine, valproic acid, lamotrigine, topiramate) *(Beniczky SA, et al., 2012).* However, this study did not measure baseline BMD before AED administration. Thus the findings of cross-sectional studies may be affected by the differences in baseline BMD amongst study subjects and by other factors such as past intake of other AEDs, physical activity, diet, and sun exposure. Contrary to this, a recent prospective study found no significant changes in BMD of the femoral neck, total femur or lumbar spine after LEV administration of mean duration 14.16 ± 3.36 months. Biochemical bone markers (calcium, phosphorus, 25-hydroxyvitamin D, alkaline phosphatase, bone alkaline phosphatase, parathyroid hormone, osteocalcin, CTX-I, insulin-like growth factor-1) also showed no significant change *(Koo DL, et al., 2013).* Traditionally, attention to the problem of AED-induced bone loss has been focused on those drugs that induce the hepatic cytochrome P450 enzyme system, thereby increasing the metabolism of vitamin D. However, the mechanisms of AED-induced bone loss appear to be

multiple, including the effects of AEDs on sex-steroid hormones *(Pack A, 2008; Isojarvi JI, et al., 2005; Rattya J, et al., 2001 and Pack AM, et al., 2011).* There is one animal study which reports a significant decrease in estradiol level after LEV administration *(Svalheim S, et al., 2008).*

In the ORX rat model estrogen was more effective in preventing ORX-induced bone loss than androgen action *(Vandenput L, et al., 2002).* This finding is supported by both animal and human data, which indicate that estrogens play a crucial role in regulating the male skeleton *(Callewaert F, et al., 2010).* Although underlying mechanisms of the effect of LEV on bone are still unclear, LEV-mediated decrease of estrogen levels could represent one of the relevant mechanisms of bone loss.

In conclusion, administration of LEV in the ORX rat model can have a negative effect on bone as judged by reduced femoral BMD, decreased serum levels of OPG (a marker of bone formation) and increased levels of CTX-I (a marker of bone resorption), but this study failed to show any change in femoral bone geometry or biomechanical bone strength. Administration of LEV in the ORX-rat model may reduce adipose tissue.

The effect of lacosamide on rat bone mass

The effect of LCM on bone tissue has not yet been investigated, except for studies in juvenile dogs (published only in the form of an abstract) *(Cornet M, et al., 2010).* The ORX rat model was used as the standard model for the induction of

osteoporosis in experimental animals *(Gentile MA, et al., 2010).*

LCM-ORX had significant loss of BMD at the left and right femur after 12 weeks when compared to the control (CON-ORX). However, no significant differences in biomechanical and geometric parameters of rat right and left femurs were observed. Evaluation of bone turnover using biochemical markers specific for both bone formation (BALP, BMP-2, P1NP, OPG, IGF-1) and bone resorption (CTX-I) was without significant difference with the exception of P1NP.

We have noticed a significant change in the P1NP, which is a marker of bone formation, which was significantly lower in LCM-ORX. To our knowledge, no studies have been published with AEDs (or with CYP2C19 inhibitors) in which changes in the level of P1NP were monitored. Some further research will be necessary to verify the role and the importance of P1NP in the diagnosis, and more precisely in the pathophysiology of AEDs-O. We have discovered only one prospective study testing the influence of LCM on BMD in gonadally-intact subjects, in which the authors claim an absence of influence of LCM on BMD *(Cornet M, et al., 2010).* However, we have monitored a significant decline of BMD at the left and right femur. We assume then that long-lasting exposure to LCM can represent a certain risk to the health of bone in the setting of gonadal insufficiency. It is complicated to determine how high the risk will be in comparison to the other AEDs.

The mechanism of the effect of LCM on bone is unclear. LCM has been shown to produce a significant effect in rodents consistent with anxiolysis: LCM increased

the suppression ratio in a conditioned emotional response test, and reduced the number of marbles buried in the marble burying assay *(Higgins GA, et al., 2009; Horcajada-Molteni MN, et al., 1999).* In rodents, physical activity prevents decrease in BMD as it does in humans, which suggests that increased physical activity could be useful in the prevention of bone mineral loss, regardless of gonadal hormone deficiency *(Horcajada-Molteni MN, et al., 1999).* Therefore reduced locomotor activity could be the factor contributing to significant decrease in LF-BMD and RF-BMD in LCM-ORX compared to CON-ORX.

The effect of topimarate on rat bone mass

There was a significant decrease in whole-body BMD and that of the femurs, but no significant changes in bone turnover markers were observed.

As far as the present authors know, the first mention of a possible risk of TPM for bone in connection with treatment with AEDs is in a study dealing with an analysis of a cohort of 96 epileptics of childhood or juvenile age. Besides other things, the authors report a significant correlation of the presence of TPM in medication with an abnormal value of BMD *(Coppola G, et al., 2009).* A possible negative effect of TPM on bone tissue is also suggested by the results of a pilot study examining the BMD of the lumbar spine and hip in 19 women receiving prophylactic TPM medication for migraine (median age was 38.7 years; average length of monotherapy with TPM, 17

months; average dose of TPM, 136 mg). In 8 female patients (53%) the T-score was abnormal, i.e. in the range of osteopenia *(Vega D, et al., 2007).* Nevertheless, the results of the study were limited, not least because of the absence of a control group. TPM, as well as zonisamide and sulthiame are all inhibitors of carbonic anhydrase. Inhibition of carbonic anhydrase is the cause of metabolic acidosis in a large number of both children and adults medicated with TPM *(Belcastro V, et al., 2010).* Besides metabolic acidosis, hyperhomocysteinemia and a deficit of vitamin B12 may exert a negative effect on bone health *(Linnebank M, et al., 2011; Anderson GD, 2004).*

The weight of the rats in the experimental group decreased in a statistically significant manner. There were significant decreases in fat mass and lean body mass. The finding is in agreement with the literature data *(Merideth Ch, 2006).* As far as the markers of bone turnover are concerned, the significance of bone turnover markers for the diagnosis of antiepileptic drug-induced osteopathy is controversial: although PHT is well-known to cause significant losses of BMD and BMC, only modest changes in the markers of bone turnover have been observed in animals *(Moro-Alvarez MJ, et al., 2009; Onodera K, et al., 2001; Valimaki MJ, et al., 1997).* Similarly, in a longitudinal study of premenopausal women treated with PHT, bone turnover markers remained unchanged after 1 year, except for a significant decline in urine N-telopeptide. This result is unclear and difficult to explain, particularly in view of the significant observed femoral neck bone loss *(Pack AM, et al., 2008).* Conflicting data exist regarding the effects of CBZ on BMD and bone turnover *(Sheth RD end Hermann BP, 2007; Pack AM, et al., 2008; Verrotti A, et al., 2002; Sato Y, et*

al., 2001). VPA in animals reduced BMD and BMC and increased bone turnover *(Moro-Alvarez MJ, et al., 2007)*; there are mixed data in humans. Some have observed that VPA monotherapy resulted in decreased BMD and increased significantly markers of both bone formation and resorption *(Verrotti A, et al., 2010; Zhang J, et al., 2010),* but in the longitudinal study of young women mentioned above, the BMD was stable and bone turnover markers remained unchanged after 1 year of VPA treatment *(Pack AM, et al., 2008).*

Data for TPM are scarce. In a cohort of long-term patients treated with TPM monotherapy, significantly lower serum levels of calcium, parathormone and bicarbonates were found. Laboratory markers of increased bone turnover were also found: an increased level of osteocalcin, bone isoenzyme of alkaline phosphatase and CTX-I *(Heo K, et al., 2011).* A Chinese study in 2010, on the other hand, reported a significantly higher level of calcium in the serum without significant differences in the serum content of alkaline phosphatase *(Zhang J, et al., 2010).*

In summary, TPM can significantly reduce BMD and body weight. Besides the issues concerning the pathogenesis of the effect of TMP on bone, further studies need to address the question as to what extent the effect found by the present authors is dependent on the dose, or on the serum levels of TPM, in order to determine if it is necessary to monitor, besides BMD, also the levels of this antiepileptic agent.

The effect of lamotrigine on rat bone mass

The treatment with LTG resulted in a significant decrease in whole-body BMD and that of the femurs. Testing of the mechanical strength of the bone tissue by means of three-point bending revealed a statistically significant decrease in the maximal load values in experimental groups versus the control group. No significant changes in bone turnover markers were observed.

LTG is an inducer of UGT enzymes. Isoenzymes of the families UGT1 and UGT2 play an important role in the metabolism of xenobiotics as well as in that of a number of endogenous substances such as steroidal hormones, hormones of the thyroid gland, fat-soluble vitamins, bilirubin and biliary acids *(Ohta T, et al., 1995).* This mechanism could be relevant to the effect of LTG on bone health. The level of LTG in the present author's study was found to be above the therapeutic range for human use: 77,74 (72,28 – 84,22) μmol/L, while that of TPM was at the lower limit of the therapeutic range for human use: 22,04 (21,64 – 22,71) μmol/L. It is therefore possible that a more marked negative effect of LTG on the bone could be associated with the relatively high level of the drug in the serum. A dose-dependent effect in connection with the effect of AEDs on bone has been reported also for phenytoin. At low doses this drug, which is typically a risk agent for bone, is reported to have an osteogenic effect *(Richard D, et al., 2002).*

The weight of the rats in the experimental groups decreased in a statistically significant manner. There were significant decreases in fat mass and lean body mass

(Merideth CH, 2006). A reduction in weight after exposure to LTG was, however, reported in both people and animals *(Daoud AS, et al., 2004; Nissen-Meyer LS, et al., 2007).* The present authors presume that the greater effect of LTG on weight as found in the present paper may be connected with the previously-mentioned difference in the serum levels of the drug.

As far as the markers of bone turnover are concerned, the significance of bone turnover markers for the diagnosis of antiepileptic drug-induced osteopathy is controversial: although PHT is well-known to cause significant losses of BMD, only modest changes in the markers of bone turnover have been observed in animals *(Moro-Alvarez MJ, et al., 2009; Onodera K, et al., 2001; Valimaki MJ, et al., 1994)*. Similarly, in a longitudinal study of premenopausal women treated with PHT, bone turnover markers remained unchanged after 1 year, except for a significant decline in urine N-telopeptide. This result is unclear and difficult to explain, particularly in view of the significant observed femoral neck bone loss *(Pack A, 2008).* Conflicting data exist regarding the effects of CBZ on BMD and bone turnover *(Sheth RD end Hermann BP, 2007; Pack AM, et al., 2008; Sato Y, et al., 2001).* VPA in animals reduced BMD and BMC and increased bone turnover *(Moro-Alvarez MJ, et al., 2009);* there are mixed data in humans. Some have observed that VPA monotherapy resulted in decreased BMD and increased significantly markers of both bone formation and resorption *(Valimaki MJ, et al., 1994; Verrotti A, et al., 2002),* but in the longitudinal study of young women mentioned above, the BMD was stable and bone turnover markers remained unchanged after 1 year of VPA treatment *(Pack AM, et*

al., 2008).

Data for LTG are scarce. So far LTG has not been shown to cause significant effects on BMD and bone turnover *(Sheth RD end Hermann BP, 2007; Pack AM, et al., 2008),* except for a significantly increased level of osteocalcin, a marker of bone formation, referred in one study *(Kim SH, et al., 2007).*

In summary, LTG can significantly reduce BMD and body weight, and impair the mechanical strength of the bone. Beside the issues concerning the pathogenesis of the effect of LTG on the bone, further studies need to address the question as to what extent the effect found by the present authors is dependent on the dose, or on the serum levels of LTG, in order to determine if it is necessary to monitor, besides BMD, also the levels of this antiepileptic agent.

In conclusion, we have confirmed a BMD reduction in all 4 medications in the tested group. The negative effect was the greatest in lamotrigine and decreased sequentially in lacosamide, topiramate and levetiracetam. Lamotrigine also caused a reduction in the mechanical strength of bone in the cervical part of the femurs.

8.3. The effect of the selected newer antidepressant drugs on rat bone metabolism

The link between depression, antidepressant use, and osteoporosis is becoming more widely understood, and there is mounting evidence for the influence of depression and antidepressants on fracture rates *(Rizzoli R, et al., 2012).* However, reports examining the relationship between SSRIs and bone mass and remodelling have yielded inconsistent results. Serotonin is a neurotransmitter that is primarily found in the gastrointestinal (GI) tract, central nervous system (CNS) and platelets. Serotonin plays an important role in mood regulation, and selective serotonin reuptake inhibitors (SSRIs) are widely used psychotropic medications prescribed for the treatment of depression and anxiety. Recent animal and in vitro studies support a role for serotonin in the regulation of bone mass and remodelling *(Feuer AJ, et al., 2015).*

For this study we have chosen selected new antidepressant drugs in which the effect on bone metabolism has not yet been sufficiently investigated. To our knowledge, this study is the first clinical research with the aim of examining the effects of these antidepressant drugs on bone metabolism in rats. Mirtazapine is a member of the noradrenergic and specific serotonergic antidepressant group (NaSSa), venlafaxine is a serotonin and noradrenaline reuptake inhibitor (SNRI), and

trazodone is a selective serotonin reuptake inhibitor (SSRI).

The effect of mirtazapine on rat bone mass

We discovered that mirtazapine, which is a norepinephrine and specific serotonergic antidepressant (NaSSA), has a negative influence on BMD and reduces the mechanical strength of bones in the femoral neck region. In rodents, physical activity prevents decrease in BMD as it does in humans, which suggests that increased physical activity could be useful in the prevention of bone mineral loss, regardless of gonadal hormone deficiency *(Horcajada-Molteni MN, et al., 1999).* Therefore reduced locomotor activity could be the factor contributing to significant decrease in LF-BMD and RF-BMD in MIRTA-ORX compared to the ORX group.

There was a significant decrease compared to the ORX group in the levels of BALP and OPG, both of which are markers of bone formation. In addition, there was a significant increase in levels of sclerostin that inhibits activation of osteoblasts. Accordingly, our findings suggest that increased bone loss with serotonin-norepinephrine antidepressants is mediated via decreased bone formation.

Our findings for OPG, BALP and sclerostin suggest that mirtazapine may suppress bone turnover. We may assume that the reduction in OPG and BALP levels could be explained by the inhibition of osteoblast activity. In conclusion, long-term administration of mirtazapine in the ORX-rat model can have a negative effect on bone. Reduced BMD, reduced mechanical strength of bones in the femoral neck,

decreased levels of OPG and BALP (markers of bone formation), and increased levels of sclerostin may cause deterioration of the mechanical strength of the bone.

The effect of venlafaxine on rat bone mass

We found that treatment with venlafaxine, a serotonin–norepinephrine reuptake inhibitor, was associated with increased levels of CTX-I, a marker of bone resorption, without a compensatory increase in P1NP, but there were decreased levels of BALP, a marker of bone formation and mineralization. Our findings suggest that the increased rate of bone loss with venlafaxine is mediated via increased bone resorption and also decreased osteoblast differentiation. Among other things, we found increased levels of sclerostin. Thus we confirm the results of previous studies *(Shea ML, et al., 2013; Haney EM, et al., 2007; Cauley JA, et al., 2005; Richards JB, et al., 2007; Warden JS, et al., 2010; Warden JS, et al., 2008).* Serotonergic antidepressants may increase bone resorption via the serotonin transporter or other types of serotonin receptors *(Battaglino R, et al., 2004).* At the same time, inhibition of the 5-HTT may have significant damaging effects on bone mineral deposition in the growing mouse skeleton *(Warden JS, et al., 2010).*

Decreased levels of BALP (markers of bone formation) and increased levels of CTX-I and sclerostin and last but not least, reduced bone density in the diaphysis of both femurs may cause deterioration in the mechanical strength of the bone.

The effect of trazodone on rat bone mass

Trazodone is a triazolopyridine antidepressant with relatively small effects on cholinergic conduction. It is an effective antidepressant drug with a broad therapeutic spectrum, including anxiolytic and sedative effect. Although trazodone is usually referred to as an SSRI, it may have other pharmacological effects *(Albertazzi P, 2006).* Since the advent of the selective serotonin re-uptake inhibitors, there have been data concerning hormonal effects of particular relevance to women, specifically raised prolactin levels, which may vary from antidepressant to antidepressant. It has been suggested that the use of antidepressants decreases bone mineral density (BMD) more than is normal for the age and sex of patients, and increases the risk for fractures *(Laekeman G, et al., 2008).*

There was a significant decrease compared to the control group in the levels of OPG and BALP while levels of sclerostin were significantly increased. Other results *(Shea ML, et al., 2013)* have suggested that the primary effect of serotonergic antidepressants is the increase in bone resorption. Our findings for OPG, BALP and sclerostin suggest that trazodone may suppress bone turnover. We may assume that the reduction in OPG and BALP levels and increase in sclerostin levels could be explained by the inhibition of osteoblast activity.

In conclusion, the tested groups as a whole demonstrated a negative effect on bone metabolism. We confirmed a reduction of osteoblastic activity in all 3 medications. However there are differences between individual drugs in the extent of the negative effect. The greatest effect was observed in trazadone. Moreover tests of mirtazapine confirmed its effect on the brittleness of bone in the cervical region of the femurs.

9. CONCLUSION

1 a) Our set goal was to determine the effect of orchidectomy on bone metabolism in rats.

We found that after 12 weeks post-orchidectomy there was a negative effect on bone metabolism in rats. These results established these animals as suitable models for investigating androgenic modulation of body composition.

2 a) A second goal was to determine the effect of selected antiepileptic drugs (levetiracetam, lacosamide, topiramate, lamotrigine) on bone metabolism in rats.

We determined that long-term administration of levetiracetam, lacosamide and topiramate can have a negative effect as judged by reduced femoral BMD. However, after 12 weeks the results showed no reduction of biomechanical bone strength. On the other hand, as well as a reduction in BMD, long-term administration of lamotrigine resulted in impairment of the mechanical strength of the bone.

b) We also wanted to determine the extent of the (negative) effect of selected antiepileptic drugs in comparison to the control group.

We detected a negative effect in all selected antiepileptic drugs. The extent of the negative effect was greatest for lamotrigine, and decreased sequentially in lacosamide, topiramate and levetiracetam.

3 a) Another objective was to determine the effect of selected antidepressant drugs (mirtazapine, venlafaxine, trazodone) on bone metabolism in rats.

Our findings after 12 weeks suggest that administration of mirtazapine may suppress bone turnover, especially in the femoral neck. Long-term administration of venlafaxine and trazodone indicated inhibition of osteoblast activity.

b) Finally we were interested in the extent of the (negative) effect of selected antidepressant drugs in comparison to the control group.

In all the selected antidepressant drugs we determined a verifiable negative effect on bone metabolism in rats. However there were differences between the individual drugs in the extent of the negative effect. Osteoblastic

activity was impaired the most by trazodone and least by mirtazapine. Surprisingly in mirtazapine, we also confirmed the highest reduction in femoral neck mechanical resistance.

10. LITERATURE

Alam A, Voronovich Z, Carley JA. A review of therapeutic uses of mirtazapine in psychiatric and medical conditions. *Prim Care Companion CNS Disord*. 2013;15(5).

Albertazzi P. Noradrenergic and serotonergic modulation to treat vasomotor symptoms. *J Br Menopause Soc*. 2006; 12(1): 7-11.

Alghamdi HS, van den Beucken JJ, Jansen JA. Osteoporotic rat models for evaluation of osseointegration of bone implants. *Tissue Eng Part C Methods*. 2014; 20(6): 493-505.

Ali II, Herial NA, Orris M, et al. Migraine prophylaxis with topiramate and bone health in women. *Headache*. 2011; 51(4): 613-616.

Anderson GD. Pharmacogenetics and enzyme induction/ inhibition properties of antiepileptic drugs. *Neurology*. 2004; 63: 3-8.

Anwar MJ, Radhakrishna KV, Vohora D. Phenytoin and sodium valproate but not levetiracetam induce bone alterations in female mice. *Can J Physiol Pharmacol*. 2014; 92(6): 507-511.

Battaglino R, Fu J, Spate U, et al. Serotonin regulates osteoclast differentiation through its transporter. *J Bone Miner Res.* 2004; 19: 1420–1431.

Beniczky SA, Viken J, Jensen LT, et al. Bone mineral density in adult patients treated with various antiepileptic drugs. *Seizure*. 2012; 21(6), 471-472.

Belcastro V, Striano P, Gorgone G, et al. Hyperhomocysteinemia in epileptic patients on new antiepileptic drugs. *Epilepsia*. 2010; 51(2): 274-279.

Bhattoah HP, Wamwaki J, Kalina E, et al. Serum sclerostin levels in healthy men over 50 years of age. *J Bone Miner Metab*. 2013; 31(5): 579-584.

Borges NC, Barrientos-Astigarraga RE, Sverdloff CE, et al. A fast, sensitive and simple method for mirtazapine quantification in human plasma by HPLC-ESI-MS/MS. Application to a comparative bioavailability study. *Biomed. Chromatogr.* 2012; 26 (11): 1399-1407.

Bruyère O, Reginster JY. Osteoporosis in patients taking selective serotonin reuptake inhibitors: a focus on fracture outcome. *Endocrine*. 2014; 48(1), 65 – 68.

Callewaert F, Sinnesael M, Gielen E, et al. Skeletal sexual dimorphism: relative contribution of sex steroids, GH-IGF1, and mechanical loading. *J Endocrinol*. 2010; 207(2): 127-134.

Carbone LD, Johnson KC, Robbins J, et al. Antiepileptic drug use, falls, fractures, and BMD in postmenopausal women: findings from the women's health

initiative (WHI). *J Bone Miner Res*. 2010; 25(4):873-81.

Cauley JA, Fullman RL, Stone KL, Zmuda, et al. Factors associated with the lumbar spine and proximal femur bone mineral density in older men. *Osteoporos Int.* 2005; 16: 1525–1537.

Compston JE. Sex Steroids and Bone. *Physiological Reviews*. 2001; 81 (1): 419-447.

Compton JT, Lee YF. A review of osteocyte function and the emerging importance of sclerostin. *J Bone Joint Surg*. 2014; 96 (19): 1659–1668.

Coppola G, Fortunato D, Auricchio G, et al. Bone mineral density in children, adolescents, and young adults with epilepsy. *Epilepsia*. 2009; 50(9): 2140-2146.

Cornet M, Tytgat D, Léonard M. Lacosamide does not alter bone densitometry parameters in juvenile dogs. Abst. 2.191, 2010.

Daoud AS, Bataineh H, Otoom S, et al. The effect of Vigabatrin, Lamotrigine and Gabapentin on the fertility, weights, sex hormones and biochemical profiles of male rats. *Neuro Endocrinol* . 2004; 25(3):178-183.

Dooley M, Plosker GL. Levetiracetam. A review of its adjunctive use in the management of partial onset seizures. *Drugs*. 2000; 60: 871–893.

Dunitz M. Osteoporosis: Diagnosis and managment. London: Martin Dunitz LTD,1998. ISBN 1-85317-613-3.

Dunitz M. Bone markers, biomechanical and clinical perspectives. London: Martin Dunitz LTD, 2001. ISBN 1-841184-067x.

Ebrahimi F, Ebrahimitalab A, Es'haghi Z. Magnetized silane-coupling agent KH-570 based solid-phase extraction followed by gas chromatography-flame ionization detection to determine venlafaxine in human hair and aqueous environmental samples. *Arch Environ Contam Toxicol.* 2015; 68(2): 412-420.

Emerton KB, Hu B, Woo AA, et al. Osteocyte apoptosis and control of bone resorption following ovariectomy in mice. Bone. 2010; 46 (3): 577–583.

Engel J, Pedley TA. Epilepsy a comprehensive text book. USA: Lippincott Williams&Wilkins, 2008. ISBN 0-7817-5777-0.

Eskandari F, Martinez PE, Torvik S, et al. Low bone mass in premenopausal women with depression. *Arch Intern Med*. 2007; 167: 2329–2336.

Feuer AJ, Demmer RT, Thai A, et al. Use of selective serotonin reuptake inhibitors and bone mass in adolescents: An NHANES study. *Bone.* 2015; 78: 28-33.

Florencio-Silva R, da Silva Sasso GR, Sasso-Cerri E, et al. Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells. *BioMed Research International*. 2015; Epub. doi: 10.1155/2015/421746.

Gentile MA, Nantermet PV, Vogel RL, et al. Androgen-mediated improvement of body composition and muscle function involves a novel early transcriptional program including IGF-1, mechano growth factor, and induction of {beta}-catenin. *J Mol Endocrinol*. 2010; 44: 55–73.

Giannopoulou EZ, Gortner L, Peterlini S, et al. Topiramate-induced nephrolithiasis. *Clin Case Rep*. 2015; 3(6): 508-509.

Greenaway C, Ratnaraj N, Sander JW, et al. A High-performance liquid chromatography assay to monitor the new antiepileptic drug lacosamide in patients with epilepsy. *Ther Drug Monit*. 2010; 32(4): 448-452.

Halford JJ, Lapointe M. Clinical Perspectives on Lacosamide. *Epilepsy Currents*. 2009; 9(1): 1–9.

Hammad LF. Bone mineral density in university aged Saudi females. *Pak J Med Sci*. 2015; 31(3): 556–560.

Haney EM, Chan BK, Diem, SJ, et al. Association of low bone mineral density with selective serotonin reuptake inhibitor use by older men. *Arch Intern Med*. 2007; 167: 1246–1251.

Heo K, Rhee Y, Lee HW, et al. The effect of topiramate on bone mineral density and markers of bone mineral metabolism in premenopausal women with epilepsy. *Epilepsia*. 2011; 52(10): 1884-1889.

Higgins GA, Breysse N, Undzys E, et al. The anti-epileptic drug lacosamide (Vimpat) has anxiolytic property in rodents. *Eur J Pharmacol*. 2009; 624(1-3):1-9.

Horcajada-Molteni MN, Davicco MJ, Coxam V, et al. Treadmill running starting 3 months after orchidectomy restores femoral bone mass in rats. *Eur J Appl Physiol Occup Physiol*. 1999; 79(3): 251-259.

Isojarvi JI, Tauboll E, Herzog AG. Effect of antiepileptic drugs on reproductive endocrine function in individuals with epilepsy. *CNS Drugs.* 2005; 19(3): 207-223.

Johannessen LC, Larsson PG, Rytter E, et al. Antiepileptic drugs in epilepsy and other disorders -- a population-based study of prescriptions. *Epilepsy Res*. 2009; 87(1): 31-39.

Kanis JA. Osteoporosis. Oxford: Blackwell science LTD, 1994. ISBN 0-632-03811-x.

Kim SH, Lee JW, Choi KG, et al. A 6-month longitudinal study of bone mineral density with antiepileptic drug monotherapy. *Epilepsy Behav*. 2007; 10(2): 291-295.

Kini U, Nandeesh BN. Physiology of bone formation, remodeling, and metabolism, Berlin: Springer-Verlag Berlin Heidelberg, 2012. ISBN 978-3-642-02399-6.

Koo DL, Joo EY, Kim D, et al. Effects of levetiracetam as a monotherapy on bone mineral density and biochemical markers of bone metabolism in patients with epilepsy*. Epilepsy research*. 2013; 104 (1-2): 134-139.

Kousteni S, Chen JR, Bellido T,et al. Reversal of bone loss in mice by nongenotropic signaling of sex steroids. *Science*. 2002; 298 (5594): 843–846.

Laekeman G, Zwaenepoel L, Reyntens J, et al. Osteoporosis after combined use of a neuroleptic and antidepressants. *Pharm World Sci.* 2008; 30(5): 613-616.

Lancelin F, Franchon E, Kraoul L, et al. Therapeutic drug monitoring of levetiracetam by high-performance liquid chromatography with photodiode array ultraviolet detection: preliminary observations on correlation between plasma concentration and clinical response in patients with refractory epilepsy. *Therap Drug Monit*. 2007; 29(5): 576-583.

Lang DG, Wang CM, Cooper BR. Lamotrigine, phenytoin and carbamazepine interactions on the sodium current present in N4TG1 mouse neuroblastoma cells. *J Pharmacol Exp Ther.* 1993; 266: 829–885.

Lazzari AA, Dussault PM, Thakore-James M, et al. Prevention of bone loss and vertebral fractures in patients with chronic epilepsy—Antiepileptic drug and osteoporosis prevention trial. *Epilepsia.* 2013; 54(11): 1997-2004.

Linnebank M, Moskau S, Semmler A, et al. Antiepileptic drugs interact with folate and vitamin B12 serum levels. *Ann Neurol*. 2011; 69(2): 352-359.

Levy RH, Mattson RH, Meldrum BS, Perucca E. Antiepileptics drugs, fifth edition. USA: Lippincott Williams&Wilkins, 2002. ISBN 0-7817-2321-3.

Macari S, Duffles LF, Queiroz-Junio CM. Oestrogen regulates bone resorption and cytokine production in the maxillae of female mice. *Arch Oral Biol*. 2015; 60(2): 333-341.

Malakova J, Brozmanova H, Vorisek V, et al. A Capillary GC Method Using Nitrogen Phosphorus Detection for Determination of Topiramate in Patients with Epilepsy. *Chromatographia*. 2007; 66 (5/6): 363-367.

Malik P, Gasser RW, Moncayo RC, et al. Bone mineral density and bone metabolism in patients with major depressive disorder without somatic comorbidities. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. 2013; 44: 58-63.

Marcus R, Feldman D, Kelsey J. Osteoporosis. United Kingdom: Academic press., 1995. ISBN 0-12-470860-9.

Maxhimer JB, Bradley JP, Lee JC. Signaling pathways in osteogenesis and osteoclastogenesis: Lessons from cranial sutures and applications to regenerative medicine. Genes & Diseases. 2015; 2 (1): 57–68.

McCormick RK. Osteoporosis: Integrating Biomarkers and Other Diagnostic Correlates into the Management of Bone Fragility. *Altern Med Rev*. 2007; 12(2): 113-45.

Merideth CH. A single-center, double-blind, placebo-controlled evaluation of lamotrigine in the treatment of obesity in adults. *J Clin Psychiatry*. 2006 Feb;67(2):258-62.

Michelhaugh SK, Basha M, Rhoney DH, et al. Acute or chronic use of lacosamide does not alter its distribution between serum and cerebrospinal fluid. *Epilepsia.* 2015. doi: 10.1111/epi.13111. epub

Moro-Alvarez MJ, Díaz Curiel M, de la Piedra C, et al. Bone disease induced by phenytoin therapy: clinical and experimental study. *Eur Neurol*. 2009; 62(4): 219-230.

Nagy V, Penninger JM. The RANKL-RANK Story. *Gerontology.* 2015; DOI: 10.1159/000371845 Epub.

Nissen-Meyer LS, Svalheim S, Taubøll E, et al. Levetiracetam, phenytoin, and valproate act differently on rat bone mass, structure, and metabolism. *Epilepsi*a. 2007; 48(10):1850-1860.

Ohta T, Wergedal JE, Matsuyama T, et al. Phenytoin and fluoride act in concert to stimulate bone formation and to increase bone volume in adult male rats. *Calcif Tissue Int.* 1995; 56: 390-397.

Onodera K, Takahashi A, Mayanagi H, et al. Phenytoin-Induced Bone Loss and Its Prevention with Alfacalcidol or Calcitriol in Growing Rats. *Calcif Tissue Int*. 2001 69: 109-116.

Pack A. Bone health in people with epilepsy: is it impaired and what are the risk factors? *Seizure*. 2008; 17(2): 181-186.

Pack AM, Morrell MJ, Randall A, et al. Bone health in young women with epilepsy after one year of antiepileptic drug monotherapy. *Neurology*. 2008; 70(18): 1586-1593.

Pack AM: Treatment of epilepsy to optimize bone health. *Curr Treat Options Neurol*. 2011; 13(4): 346-354.

Pack AM, Morrell MJ, McMahon DJ, et al. Normal vitamin D and low free estradiol levels in women on enzyme-inducing antiepileptic drugs. *Epilepsy Behav.* 2011; 21(4): 453-458.

Perucca E. The Clinical Pharmacokinetics of the New Antiepileptic Drugs. *Epilepsia*. 1999; 40 (9): 7-13.

Phabphal K, Geater A, Limapichat K et al. The association between CYP 2C9 polymorphism and bone health. *Seizure*. 2013; 22(9): 766-771.

Rabenda V, Nicolet D, Beaudart C, et al. Relationship between use of antidepressants and risk of fractures: a meta-analysis. *Osteoporos Int.* 2013; 24: 121–137.

Raggatt LJ, Partridge NC. Cellular and molecular mechanisms of bone remodeling. *J Biol Chem.* 2010; 285(33): 25103-25108.

Raisz LG. Physiology and Pathophysiology of Bone Remodeling. *Clinical Chemistry.*1999; 45(8): 1353-1358.

Rattya J, Pakarinen AJ, Knip M. Early hormonal changes during valproate or carbamazepine treatment: a 3-month study. *Neurology.* 2001; 57(3): 440-444.

Reimers A. New antiepileptic drugs and women. *Seizure*. 2014; 23 (8): 585 – 591.

Richard D, Picard F, Lemieux C, et al. The effects of topiramate and sex hormones on energy balance of male and female rats. *Int J Obes Relat Metab Disord.* 2002; 26 (3): 344-353.

Richards JB, Papaioannou A, Adachi JD, et al. Effect of selective serotonin reuptake inhibitors on the risk of fracture. *Arch Intern Med*. 2007; 167: 188–194.

Rizzoli R, Cooper C, Reginster JY, et al. Antidepressant medications and

osteoporosis. *Bone*. 2012; 51(3): 606-13.

Roggia C, Gao Y, Cenci S, et al. Up-regulation of TNF-producing T cells in the bone marrow: a key mechanism by which estrogen deficiency induces bone loss in vivo. *Proc Natl Acad Sci U S A.* 2001; 98 (24): 13960 – 13965.

Ryu SJ, Ryu DS, Kim JY, et al. Bone Mineral Density Changes after Orchiectomy using a Scrotal Approach in Rats. *Korean J Spine*. 2015; 12(2): 55-59.

Sato Y, Kondo I, Ishida S, et al. Decreased bone mass and increased bone turnover with valproate therapy in adults with epilepsy. *Neurology.* 2001; 57(3): 445-449.

Schoppet M, Preissner KT, Hofbauer LC. RANK ligand and osteoprotegerin: paracrine regulators of bone metabolism and vascular function. Arterioscler. Thromb. Vasc. Biol. 2002; 22 (4): 549–553.

Schürer C, Wallaschofski H, Nauck M, et al. Fracture Risk and Risk Factors for Osteoporosis: Results From Two Representative Population-Based Studies in North East Germany (Study of Health in Pomerania: SHIP-2 and SHIP-Trend). *Dtsch Arztebl Int*. 2015; 112(21-22): 365-71.

Seibel MJ. Biochemical markers of bone turnover: part I: biochemistry and variability. *Clin biochem Rev.* 2005; 26(4): 97-122.

Shah AD, Shoback D, Lewiecki EM. Sclerostin inhibition: a novel therapeutic approach in the treatment of osteoporosis. *Int J Womens Health*. 2015; 7: 565-80.

Shea ML, Garfield LD, Teitelbaum S, et al. Serotonin-norepinephrine reuptake inhibitor therapy in late-life depression is associated with increased marker of bone resorption. *Osteoporosis Int*. 2013; 24: 1741 – 1749.

Sheth RD, Hermann BP. Bone mineral density with lamotrigine monotherapy for epilepsy. *Pediatr Neurol*. 2007; 37(4): 250-254.

Svalheim S, Tauboll E, Surdova K, et al. Long-term levetiracetam treatment affects reproductive endocrine function in female Wistar rats. *Seizure*. 2008; 17: 203-209.

Shea ML, Garfield LD, Teitelbaum S, et al. Serotonin-norepinefrine reuptsake inhibitor therapy in late-life depression is associated with increased marker of bone resorption. *Osteoporos Int.* 2013; 24(5): 1741-1749.

Svalheim S, Røste LS, Nakken KO, et al. Bone health in adults with epilepsy. *Acta Neurol Scand Suppl.* 2011; 191: 89-95.

Valimaki MJ, Tiihonen M, Laitinen K. Bone mineral density measured by dual-energy x-ray absorptiometry and novel markers of bone formation and resorption in patients on antiepileptic drugs. *J Bone Miner Res*. 1997; 9(5): 631-637.

Vandenput L, Boonen S, Van Herck E, et al. Evidence from the aged

orchidectomized male rat model that 17beta-estradiol is a more effective bone-sparing and anabolic agent than 5alpha-dihydrotestosterone. *J Bone Miner Res*. 2002; 17(11): 2080-2086.

Vanderschueren D, Vandenput L, Boonen S, et al. An aged rat model of partial androgen deficiency: prevention of both loss of bone and lean body mass by low-dose androgen replacement. *Endocrinology*. 2000; 141: 1642–1647.

Vanpee D, Laloyaux P, Gillet JB. Seizure and hyponatraemia after overdose of trazodone. *The American Journal of Emergency Medicine.* 1999; 17 (4): 430–431.

Vega D, Maalouf NM, Sakhaee K. Increased propensity for calcium phosphate kidney stones with topiramate use. *Expert Opin Drug Saf.* 2007; 6: 547-57.

Verrotti A, Greco R, Latini G, et al. Increased bone turnover in prepubertal, pubertal, and postpubertal patients receiving carbamazepine. *Epilepsia*. 2002; 43(12): 1488-1492.

Verrotti A, Agostinelli S, Coppola G, et al. A 12-month longitudinal study of calcium metabolism and bone turnover during valproate monotherapy. *Eur J Neurol*. 2010; 17(2): 232-237.

Walker MC and Sander JW. New anti-epileptic drugs. *Expert Opin Investig Drugs*. 1999; 8: 1497–1510.

Walsh MC, Choi Y. Biology of the RANKL–RANK–OPG system in immunity, bone, and beyond. *Front Immunol*. 2014; 20(5): 511.

Warden JS, Nelson IR, Fuchs RK, et al. Serotonin (5-hydroxytryptamine) transporter inhibition causes bone loss in adult mice independently of estrogen deficiency. Menopause. 2008; 15 (6): 1176-1183.

Warden JS, Roblingb AG, Haneyc EM, et al. The emerging role of serotonin (5-hydroxytryptamine) in the skeleton and its mediation of the skeletal effects of low-density lipoprotein receptor-related protein 5 (LRP5). *Bone.* 2010; 46(1): 4 – 12.

Wheater G, Elshahaly M, Tuck SP, et al. The clinical utility of bone marker measurements in osteoporosis. *J Transl Med*. 2013; 29 (11): 201.

Weilbaecher KN, Guise TA, McCauley LK. Cancer to bone: a fatal attraction. *Nature Reviews Cancer. 2011;* 11: 411-425.

Wu Q, Bencaz AF, Hentz JG, et al. Selective serotonin reuptake inhibitor treatment and risk of fractures: a meta-analysis of cohort and case-control studies. Osteoporos Int. 2012;23(1):365-75.

Wu Q, Qu W, Crowell MD, et al. Tricyclic antidepressant use and risk of fractures: a meta-analysis of cohort and case-control studies. J Bone Miner Res. 2013; 4: 753-63.

Zhang J, Wang KX, Wei Y, et al. Effect of topiramate and carbamazepine on bone metabolism in children with epilepsy. *Zhongguo Dang Dai Er Ke Za Zhj*. 2010; 12(2): 96-98.

Zhang L, Xie WW, Li LH, et al. Efficacy and safety of prolonged-release trazodone in major depressive disorder: a multicenter, randomized, double-blind, flexible-dose trial. *Pharmacology*. 2014; 94: 199–206.

Zhaoxia LI, Zhuanglei GAO, Chengjuan JIN, et al. The Efficacy of Leviteracetam versus Carbamazepine for Epilepsy: A Meta-Analysis. *Iran J Public Health*. 2014; 43(12): 1616–1626.

11. SUPPLEMENTS (Selected papers, published by our group)

11.1.Appendix1

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The effect of levetiracetam on rat bone mass, structure and metabolism

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Summary
Objective: To determine the effect of levetiracetam (LEV) Lon bone mineral density (BMD), mineral content (BMC), bone markers, body composition and bone mechanical strength in the orchidectomised (ORX) rat model.
Method: 16 orchidectomised Wistar rats were divided into control and test groups, 8 rats in each group. The control rats received standard laboratory diet (SLD) while rats in the test group were fed with SLD enriched with LEV for 12 weeks. BMD was measured by dual energy X-ray absorptiometry at the whole body, lumbar spine and femur. Bone marker concentrations were examined of osteoprotegerin (OPG) and insulin-like growth factor 1 (IGF-1) in serum, and amino-terminal propeptide of procollagen type I (PINP), carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), bone alkaline phosphatase (ALPL), and bone morphogenetic protein 2 (BMP-2) in bone homogenate. The femurs were used for biomechanical testing.
Results: Compared to the control group we found lower fat mass, lower BMD in the area of the left femur, lower BMC in both femurs, a reduced concentration of OPG, and an increased concentration of CTX-I of borderline statistical significance ($p = 0.0661$). Biomechanical parameters did not differ between groups.
Conclusions: Significant loss of BMD or BMC was seen at the left and right femur area in the LEV group. Administration of LEV in the ORX-rat model significantly decreased levels of OPG (marker of bone formation) in serum and increased levels of CTX-I (marker of bone resorption) in bone homogenate, but results in this study did not reveal any change in biomechanical bone strength. Administration of LEV in the ORX-rat model may reduce adipose tissue. Further studies in animals and humans will be needed to confirm these findings.

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Introduction

Epilepsy is a condition affecting 0.5—1% of the population in developed countries. The disease is often chronic and lifelong treatment may be required. Antiepileptic drugs (AEDs) can adversely affect bone health. A number of theories have been proposed to explain why AEDs affect bone, but none explains all the reported effects. Cytochrome P450 enzyme-inducing AEDs are most commonly associated with negative impact on bone, but studies also suggest an effect of valproate (Pack, 2008). There is limited evidence that also use of newer AEDs may have adverse effects on bone mineral density (BMD) and metabolism (Heo et al., 2011; Ali et al., 2011; Takahashi et al., 2003; Babacan et al., 2012; Cansu et al., 2008; Mintzer et al., 1996; Ensrud et al., 2008).

Levetiracetam (LEV), (*S*)-α-ethyl-2-oxo-1-pyrrolidine acetamide, an analog of piracetam, is a relatively new broad-spectrum AED with a favorable tolerability and efficacy profile and a low potential for drug interactions. LEV is used in treating partial, generalized and myoclonic seizures. Despite wide therapeutic use of LEV, to our knowledge there is only one animal study, which reports changes in biomechanical strength properties of femoral bones from rats, along with documentation of changes in BMD and biochemical markers of bone turnover. This study demonstrates a biphasic dose-dependent effect of LEV on biomechanical bone strength, which may be related to microstructural changes in bone matrix (Nissen-Meyer et al., 2007).

There is only limited and conflicting data concerning the effect of LEV on bone health in humans (Beniczky et al., 2012; Koo et al., 2013).

We report here our findings in ORX-rats fed on LEV-enriched diet for 12 weeks concerning the impact of LEV on bone mineral density (BMD), content (BMC), bone metabolism markers, and biomechanical properties.

Methods

Animals

All animals received humane care in accordance with the guidelines set by the institutional Animal Use and Care Committee of Charles University, Prague, Faculty of Medicine in Hradec Kralove, Czech Republic. The protocol of the experiment was approved by the same committee. The experiments used eight-week-old male albino Wistar rats (Biotest s.r.o., Konarovice, Czech Republic). The animals were hosted in groups of 4 in plastic cages. During the experimental period the animals were maintained at controlled conventional conditions (12 h light and 12 h dark, temperature 22 ± 2 °C, air humidity 30—70%). Tap water and standard laboratory diet (SLD, VELAS, a.s., Lysa nad Labem, Czech Republic) or SLD enriched with LEV were given *ad libitum*. The weights of the rats were monitored once a week.

Experiment

Rats weighing (270 ± 7 g) at the beginning of the experiment were divided into two groups of 8 animals:

1. CON-R: orchidectomised control fed with SLD
2. LEV-R: orchidectomised rat fed with SLD enriched with LEV (101 mg/25 g of the diet; Levetiracetam, UCB Pharma)

At the beginning of the experiment the rats (CON-R and LEV-R) underwent bilateral orchidectomy under ether anesthesia. On the second day after operation the LEV-R began to receive SLD enriched with LEV and the CON-R only SLD, both diets *ad libitum*. After 12 weeks, the animals were sacrificed by blood withdrawal from the abdominal aorta under ether anesthesia, and the obtained serum was aliquoted and stored at −80 °C for ensuing biochemical analyses. After sacrifice of the rats, both tibiae and femurs were dissected free of soft tissue, wrapped in gauze moistened with saline and frozen to −80 °C till required for analysis.

Analysis of serum and bone homogenates

Blood serum levels of osteoprotegerin (OPG) and insulin-like growth factor 1 (IGF-1) were determined using the ELISA (Enzyme-Linked Immunosorbent Assay) method. Blood serum levels also of levetiracetam were determined in the middle and at the end of the experiment. Concentrations of levetiracetam in the samples were determined by the modified high-performance liquid chromatography method with UV photodiode-array detection (Lancelin et al., 2007). Levetiracetam and internal standard UCB 17025 were extracted after alkalinization of the sample (0.05 mL) into dichloromethane. Organic solvent was evaporated and the residue was dissolved and injected for HPLC analysis. Compounds were separated on a Zorbax SB-C8 column (Agilent Technologies, USA) at flow rate 1.1 mL/min. The mobile phase was composed of 10% acetonitrile, 7% methanol and 83% of a 20 mM phosphate buffer pH 6.7 with 0.1% triethylamine. UV detection was performed at a wavelength of 200 nm.

Bone homogenate was prepared from the tibiae. After animal sacrifice, both tibiae were carefully excised; after removal of all the surrounding skin, muscle and other soft tissue, they were stored at −80 °C until required. The proximal part of the bone (0.1 g) was disrupted and homogenized in 1.5 ml of phosphate buffer (PBS, PAA Laboratories GmbH, Pasching, Austria) with MagNA Lyser instrument (Roche Applied Science, Germany) at 6500 rpm for 20 s, and cooled on the MagNA Lyser Cooling Block. This procedure was repeated three times. The raw tissue homogenate was centrifuged at 10,000 g at 4 °C for 10 min, and the resulting supernatant was collected and stored at −80 °C.

Levels of the markers carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), amino-terminal propeptide of procollagen type I (PINP), bone alkaline phosphatase (ALPL) and bone morphogenetic protein 2 (BMP-2) were determined in this bone homogenate, also using the ELISA method.

Bone-marker levels were determined using kits from the firm Uscn Life Science Inc., Wuhan, China (PINP, Procollagen I N-Terminal Propeptide, pg/mL; OPG, Osteoprotegerin, pg/mL; IGF-1 Insulin Like Growth Factor 1, pg/mL; CTX-I, Cross Linked C-Telopeptide Of Type I Collagen; pg/mL; ALPL,

Alkaline Phosphatase, Liver/Bone/Kidney, ng/ml; BMP-2, Bone Morphogenetic Protein 2, pg/mL).

Dual energy X-ray absorptiometry analysis

The rat bone mineral density (BMD, g/cm^2) was measured by means of dual energy X-ray absorptiometry (DEXA) on a Hologic Delphi A device (Hologic, MA, USA) at the Osteocentre of the Faculty Hospital Hradec Kralove, Czech Republic. Before measurements, a tissue calibration scan was performed with the Hologic phantom for the small animal. Bone mineral densities of the whole body, in the area of the lumbar vertebrae, and in the area of the femur were evaluated by computer using the appropriate software program for small animals (DEXA; QDR-4500A Elite; Hologic, Waltham, MA, USA). All animals were scanned by the same operator.

Biomechanical testing procedure

Mechanical testing of the rat femoral shaft and femoral neck was done with a special electromechanical custom-made testing machine (Martin Kosek & Pavel Trnecka, Hradec Kralove, Czech Republic) according to our described methods in report (Gradosova et al., 2011). For the three-point bending test, the femur was placed on a holding device with the two support points 18 mm apart. A small stabilizing preload to 10 N was applied in the anteroposterior direction to fix the bone between the contacts. A constant deformation rate of 6 mm/min was generated until maximal load failure and breaking strength (maximum load, N) were recorded. When the bone was broken, the thickness of the cortical part of the bone was measured by means of a sliding micrometer (OXFORD 0-25MM 30DEG POINTED MICROMETER, Victoria Works, Leicester, Great Britain). The proximal part of the femur was used for compression test of the femoral neck. The diaphysis of the bone was embedded into a container using a methacrylate resin, and a vertical load was applied to the top of the femoral head. A small stabilizing preload to 10 N was applied and increased at a constant speed of 6 mm/min until failure of the femoral neck. The breaking strength (maximum load, N) was recorded by the measuring unit (Digitalanzeiger 9180, Burster praezisiosmesstechnik gmbh & co kg, Gernsbach, Germany). All bones were analyzed by the same operator.

Statistical analysis

Statistical analysis was performed using the program NCSS 2007 (Number Cruncher Statistical System, Kaysville, Utah, USA). The results are of measurements made after 12 weeks of the experiment, and are presented as the median and the 25th and 75th percentiles.

Results

Body weight and composition and tissue weights

All rats showed normal weight gain during the treatment period of 3 months. The weight of the experimental group decreased, but it is statistically insignificant *versus* the control group. DXA revealed that the experimental group showed significantly decreased fat mass *versus* the control group (g, %). There were no significant differences in lean body mass between the experimental group and the control group (Table 1). The concentrations of LEV correspond to human therapeutic levels of the drug.

Levels of bone markers

Bone marker levels both in serum and from specimens of the proximal tibia were measured to assess the effects of levetiracetam treatment on bone formation. As shown in Table 2, levetiracetam administration for 12 weeks caused a significant decrease in OPG and a borderline-significant increase in CTX-I. PINP and ALPL were also increased but without statistical significance.

Dual energy X-ray absorptiometry

Bone mineral density was determined by dual energy X-ray absorptiometry after 12 weeks levetiracetam administration. Using densitometric measurements, we found loss of bone mineral density and bone mineral content of the right and left femur compared with control groups. There was no statistically significant difference in whole-body BMD or BMC between the study groups (Table 3).

Biomechanical properties

To assess the effects of levetiracetam on bone metabolism, we examined the impact of levetiracetam on bone mechanical characteristics. The measured biomechanical and geometric parameters are shown in Table 4. There was no statistically significant difference in these parameters between rats receiving levetiracetam and control rats.

Discusion

In this study in the ORX-rat model, we found that long-term LEV treatment significantly reduced BMD and BMC of the left femoral diaphysis, and BMC of the right femoral diaphysis. Biochemical markers of bone metabolism comprise markers both for bone formation (ALPL, BMP-2, PINP, OPG, IGF-1) and for bone resorption (CTX-I). We observed only a significant fall in OPG, and an increase in CTX-I of borderline statistical significance ($p = 0.0661$). No significant differences in biomechanical or geometric parameters of rat right and left femurs were observed. Adipose tissue significantly decreased in both absolute and relative terms, while body weight decreased at borderline statistical significance ($p = 0.063$).

There are only a few and conflicting data in the literature concerning the effect of LEV on BMD. Nissen-Meyer and co-workers have reported in rats that LEV did not alter bone mass as judged by unaltered BMC and BMD. That paper suggests a dose-dependent (biphasic) effect of LEV on bone. Only low dose LEV (serum concentration 122 ± 41 umol/l) was associated with reduced biomechanical strength and reduced levels of serum osteocalcin, a marker of bone

formation, but not high dose (serum LEV concentration 277 ± 65 umol/l). Their results suggest LEV may have a harmful effect on trabecular bone rather than cortical bone, which would be manifested in a change in BMD. Compared to this previous study (Nissen-Meyer et al., 2007), our results suggest that in the ORX-rat model LEV may affect bone mass of the femoral diaphysis (cortical bone). The serum LEV concentration in our study (201.62 [191.9025—217.815] umol/l) was comparable to that reported in the high-dose group in the previous study (Nissen-Meyer et al., 2007). However, because LEV was administered in different ways (SLD enriched with LEV *ad libitum* in the present study *versus* twice-daily gastric feeding in the Nissen-Meyer paper), and since we don't know the timing of blood sampling relative to drug administration in Nissen-Meyer's paper, there is limited validity in comparing plasma concentrations of LEV between the two studies. Osteoprotegerin (OPG) functions as a soluble decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL), and acts by competing with receptor activator of nuclear factor kappa-B (RANK), which is expressed on osteoclasts for specifically binding to RANKL. RANKL is produced by osteoblastic lineage cells and promotes osteoclast activation leading to enhanced bone resorption and bone loss. OPG, which is secreted by osteoblastic cells, prevents RANKL interaction and subsequent stimulation with its receptor RANK (Gradosova et al., 2011). CTX-I is released from the C-terminal part of telopeptide of collagen I by proteolytic enzymes during its degradation into the systemic circulation, and it is thus a sensitive marker of bone resorption. Our results (in regard to OPG and CTX-I levels) suggest that LEV may suppress bone turnover. We may assume that the reduction in serum OPG levels could be explained by the inhibition of osteoblast activity.

In research on humans, there have been conflicting reports on bone strength and metabolism. One retrospective cross-sectional study suggested that LEV is more often associated with decreased BMD compared to the other AEDs (carbamazepine, valproic acid, lamotrigine, topiramate) (Beniczky et al., 2012). However, this study did not measure baseline BMD before AED administration. Thus the findings of cross-sectional studies may be affected by the differences in baseline BMD amongst study subjects and by other factors such as past intake of other AEDs, physical activity, diet and sun exposure. Contrary to this, a recent prospective study found no significant changes in BMD of the femoral neck, total femur or lumbar spine after LEV administration of mean duration 14.16 ± 3.36 months. Biochemical bone markers (calcium, phosphorus, 25-hydroxyvitamin D, alkaline phosphatase, bone alkaline phosphatase, parathyroid hormone, osteocalcin, CTX-I, insulin-like growth factor-1) also showed no significant change (Koo et al., 2013). Traditionally, attention to the problem of AED-induced bone loss has been focused on those drugs that induce the hepatic cytochrome P450 enzyme system, thereby increasing the metabolism of vitamin D. However, the mechanisms of AED-induced bone loss appear to be multiple, including the effects of AEDs on sex-steroid hormones (Pack, 2008; Isojarvi et al., 2005; Rattya et al., 2001; Pack et al., 2011). There is one animal study which reports a significant decrease in estradiol level after LEV administration (Svalheim et al., 2008).

In the ORX rat model estrogen was more effective in preventing ORX-induced bone loss than androgen action (Vandenput et al., 2002). This finding is supported by both animal and human data, which indicate that estrogens play a crucial role in regulating the male skeleton (Callewaert et al., 2010). Although underlying mechanisms of the effect of LEV on bone are still unclear, LEV-mediated decrease of estrogen levels could represent one of the relevant mechanisms of bone loss.

There are several limitations that should be considered in evaluating the present study. Firstly, the three-month follow-up period may be too short for monitoring changes in biomechanical properties of the bone tissue. Secondly, the sample size was small: although statistical significance is evident, the capacity to identify possible variables of confusion is limited. Finally, behavioral activity was neither controlled nor assessed. It would be interesting to compare the effect of LEV on bone against phenytoin (PHT) or against other enzyme-inducing antiepileptics, because a recent clinical report suggests that switching from PHT to LEV can significantly increase BMD and vitamin D level (Phabphal et al., 2013). In conclusion, administration of LEV in the ORX-rat model can have a negative effect on bone as judged by reduced femoral BMD, decreased serum levels of OPG (a marker of bone formation) and increased levels of CTX-I (a marker of bone resorption), but this study failed to show any change in femoral bone geometry or biomechanical bone strength. Administration of LEV in the ORX-rat model may reduce adipose tissue. Further studies in animals and humans will be needed to confirm these findings.

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References

Pack, A., 2008. Bone health in people with epilepsy: is it impaired and what are the risk factors? Seizure 17, 181—186.

Heo, K., Rhee, Y., Lee, S.A., et al., 2011. The effect of topiramate on bone mineral density and markers of bone mineral metabolism in premenopausal women with epilepsy. Epilepsia 52 (10), 1884—1889.

Ali, I.I., Herial, N.A., Orris, M., et al., 2011. Migraine prophylaxis with topiramate and bone health in women. Headache 51 (4), 613—616.

Takahashi, A., Onodera, K., Kamei, J., Sakurada, S., Shinoda, H., Miyazaki, S., Saito, T., Mayanagi, H., 2003. Effect of chronic administration of zonisamide, an antiepileptic drug, on bone mineral density and their prevention with alfacalcidol in growing rats. J. Pharm. Sci. 91, 313—318.

Babacan, O., Karaoglu, A., Vurucu, S., Yesilkaya, E., Yesilyurt, O., Cayci, T., Gulgun, M., Unay, B., Akın, R., Ozcan, O., 2012. May long term oxcarbazepine treatment be lead to secondary hyperparathyroidism? J. Clin. Neurol. 8, 65—68.

Cansu, A., Yesilkaya, E., Serdaroğlu, A., Hirfanoğlu, T.L., Camurdan, O., Gülbahar, O., Gücüyener, K., Cinaz, P., 2008. Evaluation of bone turnover in epileptic children using oxcarbazepine. Pediatr. Neurol. 39, 266—271.

Mintzer, S., Boppana, P., Toguri, J., DeSantis, A., 1996. Vitamin D levels and bone turnover in epilepsy patients taking carbamazepine or oxcarbazepine. Epilepsia 47, 510—515.

Ensrud, K.E., Walczak, T.S., Blackwell, T.L., Ensrud, E.R., Barrett-Connor, E., Orwoll, E.S., 2008. Antiepileptic drug use and rates of hip bone loss in older men: a prospective study. Neurology 71, 723—730.

Nissen-Meyer, L.S., Svalheim, S., Taubřll, E., Reppe, S., Lekva, T., Solberg, L.B., Melhus, G., Reinholt, F.P., Gjerstad, L., Jemtland, R., 2007. Levetiracetam, phenytoin, and valproate act differently on rat bone mass, structure, and metabolism. Epilepsia 48 (10), 1850—1860.

Beniczky, S.A., Viken, J., Jensen, L.T., Andersen, N.B., 2012. Bone mineral density in adult patients treated with various antiepileptic drugs. Seizure 21 (6), 471—472.

Koo, D.L., Joo, E.Y., Kim, D., Hong, S.B., 2013. Effects of levetiracetam as a monotherapy on bone mineral density and biochemical markers of bone metabolism in patients with epilepsy. Epilepsy Res. 104 (1-2), 134—139.

Lancelin, F., Franchon, E., Kraoul, L., Garciau, I., Brovedani, S., Tabaouti, K., Landré, E., Chassoux, F., Paubel, P., Piketty, M., 2007. Therapeutic drug monitoring of levetiracetam by high-performance liquid chromatography with photodiode array ultraviole detection: preliminary observations on correlation between plasma concentration and clinical response in patient with refractory epilepsy. Ther. Drug Monit. 29 (5), 576—583.

Gradosova, I., Zivna, H., Svejkovska, K., Palicka, V., Tichy, M., Zivny, P., 2011. The role of atorvastatin in bone metabolism in male albino Wistar rats. Pharmazie 66, 606—610.

Isojarvi, J.I., Tauboll, E., Herzog, A.G., 2005. Effect of antiepileptic drugs on reproductive endocrine function in individuals with epilepsy. CNS Drugs 19 (3), 207—223.

Rattya, J., Pakarinen, A.J., Knip, M., 2001. Early hormonal changes during valproate or carbamazepine treatment: a 3-month study. Neurology 57 (3), 440—444.

Pack, A.M., Morrell, M.J., McMahon, D.J., Shane, E., 2011. Normal vitamin D and low free estradiol levels in women on enzyme-inducing antiepileptic drugs. Epilepsy Behav. 21 (4), 453—458.

Svalheim, S., Tauboll, E., Surdova, K., et al., 2008. Long-term levetiracetam treatment affects reproductive endocrine function in female Wistar rats. Seizure 17, 203—209.

Vandenput, L., Boonen, S., Van Herck, E., Swinnen, J.V., Bouillon, R., Vanderschueren, D., 2002. Evidence from the aged orchidectomized male rat model that 17 beta-estradiol is a more effective bone-sparing and anabolic agent than 5 alpha-dihydrotestosterone. J. Bone Miner. Res. 17 (11), 2080—2086.

Callewaert, F., Sinnesael, M., Gielen, E., Boonen, S., Vanderschueren, D., 2010. Skeletal sexual dimorphism: relative contribution of sex steroids, GH-IGF1, and mechanical loading. J. Endocrinol. 207 (2), 127—134.

Phabphal, K., Geater, A., Limapichat, K., Sathirapanya, P., Setthawatcharawanich, S., Leelawattana, R., 2013. Effect of switching hepatic enzyme-inducer antiepileptic drug to levetiracetam on bone mineral density, 25 hydroxyvitamin D, and parathyroid hormone in young adult patients with epilepsy. Epilepsia 54 (6), 94—98.

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ERRATUM

Erratum to ''The effect of levetiracetam on rat bone mass, structure and metabolism'' [Epilepsy Res. 107 (2013) 56–60]

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The publisher regrets that Fig. 1 and Tables 1–4 were omitted from the above published article. Please find the figure and tables below:

The publisher would like to apologise for any inconvenience caused.

Table 1 Weight, fat mass and serum levetiracetam concentration after 90 days of drug expozure.

Parameter	Dose LEV ($n = 8$)	Control group ($n = 8$)	p-Value
Initial body weight (g)	274.5 [266.25–286.5]	282 [264.25–291.25]	0.741
Final body weight (g)	486.5 [472.75–490.5]	523 [493.25–538]	0.063
Fat (g)	71.35 [63.825–78.275]	90.3 [83.6–126.725]	0.0087
Fat (%)	17.05 [15.8–18.95]	22.05 [20.9–26.025]	0.0112
Lean body mass (g)	335.4 [330.8–349.05]	326.15 [312.825–374.875]	0.9864
Serum drug conc. (μmol/l)	201.62 [191.9025–217.815]	–	–

Data are expressed as median (25th–75th percentiles).

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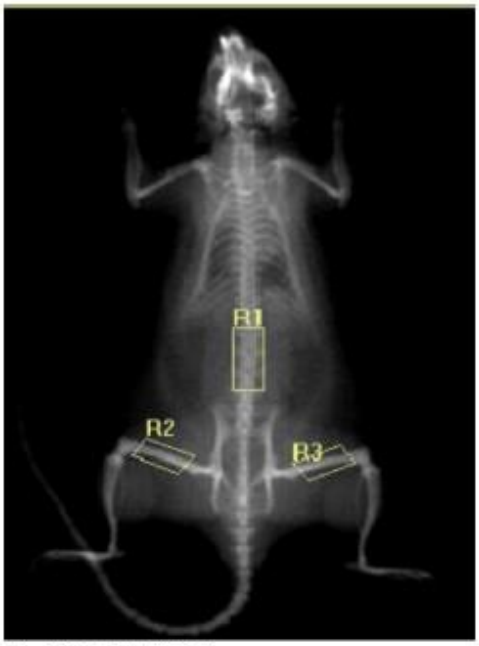

R1 – lumbar columna (L3-L5);
R2 – left femur;
R3 – right femur

Fig. 1 Evaluation of BMD in three areas of the rat skeleton. R1 – lumbar columna (L3–L5); R2 – left femur; R3 – right femur

Table 2 Levels of bone markers.

Parameter	Dose LEV ($n = 8$)	Control group ($n = 8$)	p-Value
ALPL (ng/ml)	1.490 [1.198–1.694]	1.161 [0.794–1.382]	0.1806
BMP2 (pg/ml)	831.553 [704.383–1005.833]	846.841 [633.905–985.989]	0.9647
CTX 1 (pg/ml)	2.473 [1.365–3.149]	0.972 [0.802–1.537]	0.0661
IGF-I (pg/ml)	1.158 [1.117–1.192]	1.107 [1.081–1.167]	0.8158
OPG (pg/ml)	49.628 [48.917–51.040]	54.671 [51.932–57.835]	0.0186
PINP (pg/ml)	87.395 [78.751–97.267]	78.977 [73.367–81.067]	0.1979

Data are expressed as median (25th–75th percentiles).
ALPL, bone alkaline phosphatase; BMP-2, bone morphogenetic protein; CTX-I, carboxy-terminal cross-linking telopeptide of type I collagen; IGF-1, insulin-like growth factor 1; OPG, osteoprotegerin; PINP, amino-terminal propeptide of procollagen type I.

Table 3 Effect of levetiracetam (LEV) on bone densitometric parameters.

Parameter	Dose LEV ($n = 8$)	Control group ($n = 8$)	p-Value
Whole body			
BMD (g/cm^2)	0.176 [0.174–0.176]	0.175 [0.172–0.176]	0.5151
BMC (g Ca^{2+})	13.533 [13.116–13.875]	13.989 [13.431–14.363]	0.2827
Diaphysis right femur			
BMD (g/cm^2)	0.187 [0.177–0.1917]	0.195 [0.189–0.206]	0.1048
BMC (g Ca^{2+})	0.235 [0.231–0.251]	0.278 [0.256–0.289]	0.0063
Diaphysis left femur			
BMD (g/cm^2)	0.177 [0.173–0.189]	0.195 [0.194–0.199]	0.0181
BMC (g Ca^{2+})	0.242 [0.236–0.246]	0.2767 [0.236–0.246]	0.0026
Lumbar columna (L3–L5)			
BMD (g/cm^2)	0.212 [0.210–0.213]	0.207 [0.202–0.213]	0.3520
BMC (g Ca^{2+})	0.598 [0.593–0.603]	0.623 [0.590–0.639]	0.594

Data are expressed as median (25th–75th percentiles).
BMD, bone mineral density; BMC, bone mineral content.

Table 4 Biomechanical and geometric parameters of rat right and left femurs.

Parameter	Dose LEV ($n = 8$)	Control group ($n = 8$)	p-Value
LF length (mm)	37.02 [36.845–37.075]	37.45 [36.72–37.955]	0.6601
RF length (mm)	37.13 [36.89–37.62]	37.4 [36.8–37.69]	0.9506
LF diameter (mm)	3.65 [3.335–3.72]	3.63 [3.515–3.945]	0.2092
RF diameter (mm)	3.69 [3.525–3.75]	3.63 [3.545–3.72]	0.7519
Cortical LF thickness (mm)	0.69 [0.658–0.74]	0.72 [0.673–0.735]	0.6069
Cortical RF thickness (mm)	0.7 [0.678–0.728]	0.705 [0.673–0.728]	0.5283
Maximal load of the left femoral shaft (N)	208 [195.25–223.5]	214.5 [196.75–233.75]	0.6332
Maximal load of the right femoral shaft (N)	207 [203.5–223.25]	216 [203.75–227.5]	0.5283
Maximal load of the left femoral neck (N)	140.5 [134.25–148.25]	144 [135.5–152]	0.8945
Maximal load of the right femoral neck (N)	163.5 [151.75–171.25]	156 [136.5–171.25]	0.6546

Data are expressed as median (25th–75th percentiles).
LF, left femur; RF, right femur.

11.2. Appendix 2

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The effect of topiramate and lamotrigine on rat bone mass, structure and metabolism

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ABSTRACT

There is only limited data concerning the effect of the newer antiepileptic drugs on bone. The objective of this study was to determine the effect of topiramate (TPM) and lamotrigine (LTG) monotherapy on bone mineral density (BMD), mineral content (BMC), bone markers, body composition and bone mechanical strength in the orchidectomized (ORX) rat model.

24 orchidectomized Wistar rats were divided into control and test groups, 8 rats in each group. The control rats received standard laboratory diet (SLD) while rats in the test group were fed with SLD enriched with LTG or TPM for 12 weeks. Dual energy X-ray absorptiometry was used to measure bone mineral density. The concentrations of bone metabolism markers were assayed in bone homogenate. In addition, both femurs were measured and used for biomechanical testing.

Compared to the control group, both test groups had significantly lower weight, fat mass, whole body and femur BMD, BMC and reduced mechanical strength of bone. All of these changes were more pronounced in rats exposed to LTG.

In conclusion, both LTG and TPM significantly reduce BMD and body weight and impair mechanical strength of bone. A question arises as to the degree of dependence of the effect on the dose. Further studies are warranted to establish whether LTG and TPM may have a clinically significant effect on BMD exclusively in the model of gonadectomized rats, or whether the effect applies also in the model of gonadally intact animals, and in the respective human models.

1. Introduction

Many patients with epilepsy are required to take chronically antiepileptic drugs (AEDs). For this reason the issue of undesirable effects due to long-term medication is important. Osteopathies occurring with long-term chronic antiepileptic treatment were first noted in the late sixties [1,2]. Since that time a number of theories have been proposed to explain why AEDs affect the bone, but none explains all the reported effects [3]. Most studies of the effects of AEDs on bone tissue are cross-sectional; there are only a few longitudinal studies. There is limited evidence also that use of the newer AEDs, namely topiramate (TPM), zonisamide, oxcarbazepine and gabapentin, may have adverse effects on bone mineral density (BMD) and bone metabolism [4–9]. Current studies suggest that lamotrigine (LTG) could have limited (if any) negative impact on bone health (10–12), except in combination with valproate [13].

Decline of the sex steroids is an important risk factor of osteoporosis in the elderly. In older women as well as in older man the use of AEDs was independently associated with increased rates of bone loss [9,14]. In gonadally intact subject, no negative impact of LTG on the bone has been reported so far. We have thus selected the orchidectomized rat model (the model for androgen-deficient osteoporosis), in which higher sensitivity can be expected for detection of a possible risk of xenobiotics for bone health.

We report here our findings in orchidectomized (ORX) rats fed with TPM or LTG-enriched diet for 12 weeks, on the impact of TPM or LTG on BMD, bone mineral content (BMC), bone metabolism markers, and biomechanical properties of bone.

In the same study design we have previously reported that administration of levetiracetam can have a negative effect on bone as judged by

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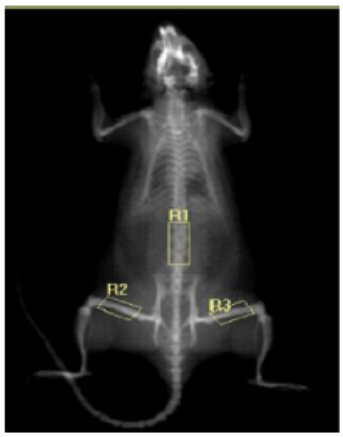

R1 – lumbar columna (L3-L5);

R2 – left femur

R3 – right femur

Fig. 1. Evaluation of BMD in three areas of the rat skeleton.

reduced femoral BMD, decreased serum levels of OPG (marker of bone formation) and increased levels of CTX-I (marker of bone resorption), but this study failed to show any change in biomechanical bone strength [15] (in the same study design, we have also observed a significant loss of BMD at the left and right femur areas in the lacosamide group, again without any change in biomechanical bone strength – publication in the peer review process).

2. Methods

2.1. Animals

All animals received humane care in accordance with the guidelines set by the Institutional Animal Use and Care Committee of Charles University, Prague, Faculty of Medicine in Hradec Kralove, Czech Republic. The protocol of the experiment was approved by the same committee. The experiments used eight-week-old male albino Wistar rats (Biotest s.r.o., Konarovice, Czech Republic). The animals were hosted in groups of 4 in plastic cages. During the experimental period the animals were maintained under controlled conventional conditions (12 h of light and 12 h of dark, temperature 22 ± 2 °C, air humidity 30–70%). Tap water, standard laboratory diet (SLD, VELAS, a.s., Lysa nad Labem, Czech Republic), SLD enriched with LTG and SLD enriched with TPM were given *ad libitum*. The weights of the rats were monitored once a week.

2.2. Experiment

The rats, weighing 270 ± 7 g at the beginning of the experiment, were divided into three groups of 8 animals:

1. CON-R: orchidectomized rats control fed with SLD;
2. LTG-R: orchidectomized rats fed with SLD enriched with LTG (39 mg/25 g of the diet; Lamotrigine, Glenmark); and
3. TPM-R: orchidectomized rats fed with SLD enriched with TPM (23 mg/25 g of the diet; Topiramat, Glenmark).

At the beginning of the experiment, the rats (CON-R, LTG-R and TPM-R) underwent bilateral orchidectomy under ether anesthesia. On the second day after operation, the LTG-R began to receive SLD enriched with LTG; the TPM-R began to receive SLD enriched with TPM; and the CON-R only SLD, both diets *ad libitum*. After 12 weeks, the animals were sacrificed by blood withdrawal from the abdominal aorta under ether anesthesia and the obtained serum was aliquoted and stored at −80 °C for ensuing biochemical analyses. After sacrificing the rats, both tibiae and femurs were dissected free of soft tissue, wrapped in gauze moistened with saline and frozen to −80 °C till the time of analysis.

2.3. Analysis of serum and bone homogenates

In the blood serum, the levels of osteoprotegerin (OPG) and insulin-like growth factor 1 (IGF-1) were determined by the ELISA (enzyme-linked immunosorbent assay) method. Concentrations of lamotrigine were measured using a modified method of high-performance liquid chromatography with UV photodiode-array detection [16]. Liquid–liquid extraction of a 0.05 ml alkalinized sample was carried out into ethyl acetate. After evaporation of the organic phase, the residue was dissolved in methanol. Lamotrigine and the internal standard BW 725C 78 were separated on a Symmetry C18 column (Waters, USA) 150 × 4.6 mm I.D., 5 μm particle size and Symmetry C18 guard column (20 × 3.9 mm I.D.). The mobile phase at isocratic flow rate of 1 ml/min contained acetonitrile (28%) and 6 mM phosphate buffer pH 6.8 (72%). The eluate was monitored at a wavelength of 306 nm. Determination of topiramate in the samples was performed using the gas chromatography-mass spectrometry method. This method was a modification of a bioanalytical method published previously [17]. The procedure included liquid–liquid extraction of 0.05 ml of the alkalinized sample with ethyl acetate. Trimethylanilinium hydroxide was used for flash methylation of topiramate and internal standard 5-(*p*-methylphenyl)-5-phenylhydantoin. Ions of m/z 352 (for the topiramate derivative) and m/z 296 (for the internal standard derivative) were recorded for data evaluation.

In the bone homogenate, the levels of the markers carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), aminoterminal propeptide of procollagen type I (PINP), bone alkaline phosphatase (BALP) and bone morphogenetic protein 2 (BMP-2) were determined, also using the ELISA method. The homogenate was prepared from the tibia. After animal sacrifice, both tibias were carefully excised; after removal of all the surrounding skin, muscle and other soft tissue, they were stored at −80 °C until required. The proximal part of the bone (0.1 g) was disrupted and homogenized in 1.5 ml of phosphate buffer (PBS, PAA Laboratories GmbH, Pasching, Austria) with a MagNA Lyser instrument (Roche Applied Science, Germany) at 6500 rpm for 20 s and cooled on a MagNA Lyser cooling block. This procedure was repeated three times.

The tissue homogenate was centrifuged at 10,000 *g* at 4 °C for 10 min. The supernatant was collected and stored at −80 °C. Kits from the firm Uscn Life Science Inc., Wuhan, China were used for

determination of the levels of bone markers (PINP, procollagen I N-terminal propeptide, pg/ml; OPG, osteoprotegerin, pg/ml; IGF-1, insulin like growth factor 1, pg/ml; CTX-I, cross linked C-telopeptide of type I collagen, pg/ml; BALP, alkaline phosphatase, liver/bone/kidney, ng/ml; BMP-2, bone morphogenetic protein 2, pg/ml).

2.4. Dual energy X-ray absorptiometry analysis

The rat bone mineral density (BMD, g/cm^2) was measured by means of dual energy X-ray absorptiometry (DEXA) on a Hologic Delphi A device (Hologic, MA, USA) at the Osteocentre of the Faculty Hospital Hradec Kralove, Czech Republic. Before measurements, a tissue calibration scan was performed with the Hologic phantom for the small animal. Bone mineral densities of the whole body, in the area of the lumbar vertebrae and in the area of the femur were evaluated (Fig. 1) by computer using the appropriate software program for small animals (DEXA; QDR-4500A Elite; Hologic, Waltham, MA, USA). All animals were scanned by the same operator.

2.5. Biomechanical testing procedure

Mechanical testing of the rat femoral shaft and femoral neck was carried out using a special electromechanical custom-made testing machine (Martin Kosek & Pavel Trnecka, Hradec Kralove, Czech Republic) according to the methods described in our report [17]. For the three-point bending test, the femur was placed on its anteroposterior direction on a holding device with the two support points 18 mm apart. A small stabilizing preload of 10 N was used to fix the bone between the contacts. A constant deformation rate of 6 mm/min was applied until load failure, and the breaking strength (maximum load, N) was recorded. When the bone was broken, the thickness of the cortical part of the bone was measured by means of a sliding micrometer (Oxford 0–25 mm 30 Deg pointed micrometer, Victoria Works, Leicester, Great Britain). The proximal part of the femur was used for compression test of the femoral neck. The load was applied to the top of the femoral head. A small stabilizing preload of 10 N was applied and then increased at a constant speed of 6 mm/min until failure of the femoral neck. The breaking strength (maximum load, N) was recorded by a measuring unit (Digitalanzeiger 9180, Burster Präzisionsmesstechnik GmbH & Co KG, Gernsbach, Germany). All bones were analyzed by the same operator.

2.6. Statistical analysis

Statistical analysis was performed using the program NCSS2007 (Number Cruncher Statistical System, Kaysville, Utah, USA). Comparison of the parameters under study employed an analysis of variance with post-hoc multiple comparison by Fisher's LSD test and Kruskal–Wallis non-parametrical analysis of variance with post-hoc multiple comparison by Dunn's test. Differences were considered significant at $p < 0.05$. Results are presented as the median and the 25th and 75th percentiles.

3. Results

3.1. Weight and body composition

The weight of both experimental groups decreased in a statistically significant manner. The weight-lowering effect was more pronounced in the LTG group than in the TPM group. There was a significant decrease in fat mass and lean body mass, again more pronounced in the LTG group (Table 1).

3.2. Dual energy X-ray absorptiometry

A significant loss of BMD was found for the whole body and the right and left femurs for both experimental groups compared with the control group, and was more pronounced for the LTG group. A loss of BMC was observed in the same parameters, but was statistically significant only for the LTG group (Table 2).

3.3. Biomechanical properties

Testing of the mechanical strength of the bone tissue by means of three-point bending revealed a statistically significant decrease in maximal load values in both experimental groups *versus* the control group; in the lamotrigine experimental group the decrease was again significantly higher than in the topiramate group *versus* the control group (Table 3). In the LTG group, cortical thickness was decreased but not in a statistically significant manner. No effect on the length of the femur was observed.

3.4. Levels of bone markers

No statistically significant difference in bone turnover markers was found (Table 4).

3.5. Serum concentrations of drugs

Serum concentrations of LTG and TPM were 77.74 (72.28–84.22) and 22.04 (21.64–22.71) μmol/l respectively. The usually-accepted therapeutic range for human use is 15–75 μmol/l for TPM and 12–66 μmol/l for LTG.

4. Discussion

This animal study has revealed that the treatment with LTG, as well as that with TPM resulted in a significant decrease in whole-body BMD and that of the femurs. The decrease in BMD was more significant in the group exposed to LTG, which showed also a significant decrease in the values of whole-body BMC and BMC of the femurs (Table 2). Testing of the mechanical strength of the bone tissue by means of three-point bending revealed a statistically significant decrease in the maximal load values in both experimental groups *versus* the control group; in the experimental group with LTG the decrease was again significantly higher than in the group with TPM (Table 3). No significant changes in bone turnover markers were observed (Table 4).

Table 1
Weight, fat mass and serum lamotrigine and topiramate concentration after 90 days of drug administration.

Parameter	Dose LTG	Dose TPM	Control group
Initial body weight (g)	273 [259.75–288.5]	274 [269.25–276.25]	282 [264.25–291.25]
Final body weight (g)	415.5 [402.25–449.25]**	486 [474.75–493]*	523 [493.25–538]
Fat (g)	64.4 [59.9–70.775]***	73.6 [67–83]*	90.3 [83.6–126.725]
Fat (%)	17.3 [17.075–18.2]*	17.15 [16.25–20.7]*	22.05 [20.9–26.025]
Lean body mass (g)	295.15 [281.85–310.8]*	334 [321.25–344.35]	326.15 [312.825–374.875]
Serum drug concentration (μmol/l)	77.74 [72.28–84.22]	22.04 [21.64–22.71]	–

Data are expressed as medians (25th–75th percentiles). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control group.

Table 2
Effect of lamotrigine and topiramate on bone densitometric parameters versus control group.

Parameter	Dose LTG	Dose TPM	Control group
Whole body			
BMD (g/cm^2)	0.168 [0.16–0.172]*	0.168 [0.166–0.169]*	0.175 [0.172–0.176]
BMC (g Ca^{2+})	12.158 [11.778–12.895]*	13.05 [12.916–13.221]	13.989 [13.431–14.363]
Diaphysis right femur			
BMD (g/cm^2)	0.169 [0.163–0.177]**	0.179 [0.176–0.188]*	0.195 [0.189–0.206]
BMC (g Ca^{2+})	0.242 [0.232–0.258]*	0.254 [0.246–0.265]	0.278 [0.256–0.289]
Diaphysis left femur			
BMD (g/cm^2)	0.174 [0.1666–0.179]**	0.179 [0.173–0.190]*	0.195 [0.194–0.199]
BMC (g Ca^{2+})	0.25 [0.247–0.264]*	0.262 [0.255–0.269]	0.2767 [0.236–0.246]
Lumbar columna (L3–L5)			
BMD (g/cm^2)	0.207 [0.201–0.210]	0.196 [0.191–0.207]	0.207 [0.202–0.213]
BMC (g Ca^{2+})	0.533 [0.516–0.593]	0.583 [0.563–0.592]	0.623 [0.590–0.639]

Data are expressed as medians (25th–75th percentiles). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control group.
BMD — bone mineral density; BMC — bone mineral content.

Since the end of the 1960s, the effect of antiepileptic agents on bone has been examined in a number of papers. However, in the case of the novel antiepileptics, data are scarce: there are no prospective studies with a sufficiently long period of monitoring. As far as the present authors know, the first mention of a possible risk of TPM for bone in connection with treatment with AEDs is in a study dealing with an analysis of a cohort of 96 epileptics of childhood or juvenile age. Beside other things, the authors report a significant correlation of the presence of TPM in medication with an abnormal value of BMD [4]. A possible negative effect of TPM on bone tissue is also suggested by the results of a pilot study examining the BMD of the lumbar spine and hip in 19 women receiving prophylactic TPM medication for migraine (median age was 38.7 years; average length of monotherapy with TPM, 17 months; average dose of TMP, 136 mg). In 8 female patients (53%) the T-score was abnormal, *i.e.* in the range of osteopenia [18]. Nevertheless, the results of the study were limited, above all because of the absence of a control group. TPM, as well as zonisamide and sulthiame are all inhibitors of carbonic anhydrase. Inhibition of carbonic anhydrase is the cause of metabolic acidosis in a large number of both children and adults medicated with TPM [19]. Acidosis promotes bone loss by osteoclast activation. However, the change of osteoclastic cell behavior in the acidosis-stimulated bone resorption process is unknown [20]. Besides metabolic acidosis, hyperhomocysteinemia (HHcy) and vitamin B12 deficiency are well-known side effects of TPM [21,22]. Homocysteine (Hcy) is known to affect bone health *via* several mechanisms, such as an increase in osteoclast activity, a decrease in osteoblast activity, and direct action of Hcy on the bone matrix. Earlier studies also revealed an alteration in bone biomechanical properties with deficiencies of vitamin B12 and folate and the degree of HHcy. Moreover, the existing data open speculation that folate and vitamin therapy act not only *via* Hcy-dependent pathways but also *via* Hcy-independent pathways [23].

The present paper observed that alteration of BMD and the mechanical strength of the bone were more marked in the group treated with LTG than in the group treated with TPM. This result is surprising to a certain degree because the literature data, in contrast to the data concerning TPM, has not yet suggested a more significant risk of LTG for the bone: in one cross-sectional and two longitudinal studies, both with a short monitoring period (6 or 12 months) no significant change in the levels of calcium, vitamin D, or BMD has been observed (10–12): either the markers of bone tissue turnover were unchanged [12], or there was a significant increase only in osteocalcin [10]. One exception relates to the combination of LTG and VPA, which in a cohort of children has been reported to have a negative effect on body height and on the marker of bone formation, osteocalcin [13]. LTG is an inducer of UGT enzymes. Isoenzymes of the families UGT1 and UGT2 play an important role in the metabolism of xenobiotics as well as in that of a number of endogenous substances such as steroidal hormones, hormones of the thyroid gland, fat-soluble vitamins, bilirubin and biliary acids [24]. This mechanism could be relevant to the effect of LTG on bone health. Unfortunately, the above-mentioned papers do not report the values of LTG levels in the serum. The level of LTG in the present authors' study was found to be above the therapeutic range for human use: 77.74 (72.28–84.22) μmol/l, while that of TPM was at the lower limit of the therapeutic range for human use: 22.04 (21.64–22.71) μmol/l. It is therefore possible that a more marked negative effect of LTG on the bone could be associated with the relatively high level of the drug in the serum. A dose-dependent effect in connection with the effect of AEDs on bone has been reported also for phenytoin. At low doses this drug, which is typically a risk agent for bone, is reported to have an osteogenic effect [25].

The weight of the rats in both experimental groups decreased in a statistically significant manner. The weight-lowering effect was more

Table 3
Biomechanical and geometric parameters of rat right and left femurs.

Parameter	Dose LTG	Dose TPM	Control group
LF length (mm)	36.78 [36.12–37.11]	37.3 [37.22–37.41]	37.45 [36.72–37.955]
RF length (mm)	37.05 [36.485–37.56]	37.26 [37.22–37.37]	37.4 [36.8–37.69]
LF diameter (mm)	3.51 [3.47–3.54]	3.57 [3.5–3.64]	3.63 [3.515–3.945]
RF diameter (mm)	3.5 [3.435–3.565]	3.52 [3.48–3.62]	3.63 [3.545–3.72]
Cortical LF thickness (mm)	0.67 [0.66–0.68]	0.75 [0.693–0.75]	0.72 [0.673–0.735]
Cortical RF thickness (mm)	0.69 [0.65–0.713]	0.74 [0.698–0.753]	0.705 [0.673–0.728]
Maximal load of the left femoral shaft (N)	180 [169.75–198]**	197.5 [192–207.75]*	214.5 [196.75–233.75]
Maximal load of the right femoral shaft (N)	186 [166.75–192]**	194.5 [188.75–201.5]*	216 [203.75–227.5]
Maximal load of the left femoral neck (N)	117 [109–120]**	139 [121.5–153–5]*	144 [135.5–152]
Maximal load of the right femoral neck (N)	119 [110–123]**	134.5 [126.5–149.75]*	156 [136.5–171.25]

Data are expressed as medians (25th–75th percentiles). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control group.
LF — left femur; RF — right femur.

Table 4
Levels of bone markers.

Parameter	Dose LTG	Dose TPM	Control group
BALP (ng/ml)	0.868 [0.838–0.963]	1.528 [1.378–1.664]	1.161 [0.794–1.382]
BMP2 (pg/ml)	817.627 [774.578–890.548]	834.857 [707.31–1057.472]	846.841 [633.90–985.989]
CTX 1 (pg/ml)	0.586 [0.53–0.675]	0.505 [0.478–0.652]	0.890 [0.763–1.280]
IGF-I (pg/ml)	1.269 [0.243–1.295]	1.111 [1.092–1.197]	1.107 [1.081–1.167]
OPG (pg/ml)	52.224 [51.102–54.545]	52.613 [50.628–53.656]	54.671 [51.932–57.835]
PINP (pg/ml)	86.84 [80.244–92.172]	87.401 [77.619–94.04]	78.977 [73.367–81.067]

Data are expressed as medians (25th–75th percentiles). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group.
BALP — bone alkaline phosphatase; BMP-2 — bone morphogenetic protein; CTX-I — carboxy-terminal cross-linking telopeptide of type I collagen; IGF-1 — insulin-like growth factor 1; OPG — osteoprotegerin; PINP — aminoterminal propeptide of procollagen type I BALP

pronounced in the LTG group than in the TPM group. There were significant decreases in fat mass and lean body mass, again more pronounced in the LTG group (Table 1). In the case of TPM, the finding is in agreement with the literature data [26]. The literature data for LTG are less consistent. A reduction in weight after exposure to LTG was, however, reported in both people and animals [27,28]. The present authors presume that the greater effect of LTG on weight as found in the present paper may be connected with the previously-mentioned difference in the serum levels of the drugs (LTG, or TPM).

As far as the markers of bone turnover are concerned, the significance of bone turnover markers for the diagnosis of antiepileptic drug-induced osteopathy is controversial: although PHT is well-known to cause significant losses of BMD and BMC, only modest changes in the markers of bone turnover have been observed in animals [29–31]. Similarly, in a longitudinal study of premenopausal women treated with PHT, bone turnover markers remained unchanged after 1 year, except for a significant decline in urine N-telopeptide. This result is unclear and difficult to explain, particularly in view of the significant observed femoral neck bone loss [12]. Conflicting data exist regarding the effects of CBZ on BMD and bone turnover [11,12,32,33]. VPA in animals reduced BMD and BMC and increased bone turnover [29]; there are mixed data in humans. Some have observed that VPA monotherapy resulted in decreased BMD and significantly increased markers of both bone formation and resorption [34,35], but in the longitudinal study of young women mentioned above, the BMD was stable and bone turnover markers remained unchanged after 1 year of VPA treatment [12].

Data for TPM and LTG are scarce. In a cohort of long-term patients treated with TPM monotherapy, significantly lower serum levels of calcium, parathormone and bicarbonates were found. Laboratory markers of increased bone turnover were also found: an increased level of osteocalcin, bone isoenzyme of alkaline phosphatase and CTX-I [5]. A Chinese study in 2010, on the other hand, reported a significantly higher level of calcium in the serum without significant differences in the serum content of alkaline phosphatase [36]. So far LTG has not been shown to cause significant effects on BMD and bone turnover [11,12], except for a significantly increased level of osteocalcin, a marker of bone formation, referred to in one study [10].

There are several limitations that should be considered in evaluating the present study. Firstly, the sample size was small: although statistical significance is evident, the capacity to identify possible variables of confusion is limited. Secondly, behavioral activity was neither controlled nor assessed — except for the pathophysiological mechanisms mentioned above the influence of LTG and/or TPM on the muscle tension or the motor activity of rats cannot be excluded [37].

In summary, this animal study reported here shows that both LTG and TPM can significantly reduce BMD and body weight, and impair the mechanical strength of the bone. Beside the issues concerning the pathogenesis of the effect of TMP/LTG on the bone, further studies need to address the question as to what extent the effect found by the present authors is dependent on the dose, or on the serum levels of LTG or TPM, in order to determine if it is necessary to monitor, besides BMD, also the levels of these antiepileptic agents. Further studies are warranted to establish whether LTG and TPM may have a clinically significant effect on BMD exclusively in the model of gonadectomized rats, or whether the effect applies also in the model of gonadally intact animals, and in the respective human models.

Finally, there is a strong need for further, particularly long term comparison prospective studies, in which the risk for bone health of all widely used AEDs can be compared.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

[1] Schmid F. Osteopathien bei antiepileptischer Dauerbehandlung. Fortschr Med 1967;85:381–2.
[2] Kruse R. Osteopathien bei antioepileptischer Langzeittherapie (vorläufige Mitteilung). Mschr Kinderheilkd 1968;116:378–80.
[3] Pack A. Bone health in people with epilepsy: is it impaired and what are the risk factors? Seizure 2008;17(2):181–6.
[4] Coppola G, Fortunato D, Auricchio G, Mainolfi C, Operto FF, Signoriello G, et al. Bone mineral density in children, adolescents, and young adults with epilepsy. Epilepsia 2009;50:2140–6.
[5] Heo K, Rhee Y, Lee HW, Lee SA, Shin DJ, Kim WJ, et al. The effect of topiramate on bone mineral density and markers of bone mineral metabolism in premenopausal women with epilepsy. Epilepsia 2011;52(10):1884–9.
[6] Takahashi A, Onodera K, Kamei J, Sakurada S, Shinoda H, Miyazaki S, et al. Effect of chronic administration of zonisamide, an antiepileptic drug, on bone mineral density and their prevention with alfacalcidol in growing rats. J Pharmacol Sci 2003;91:313–8.
[7] Mintzer S, Boppana P, Toguri J, DeSantis A. Vitamin D levels and bone turnover in epilepsy patients taking carbamazepine or oxcarbazepine. Epilepsia 2006;47:510–5.
[8] Babacan O, Karaoglu A, Vurucu S, Yesilkaya E, Yesilyurt O, Cayci T, et al. May long term oxcarbazepine treatment be lead to secondary hyperparathyroidism? J Clin Neurol 2012;8:65–8.
[9] Ensrud KE, Walczak TS, Blackwell TL, Ensrud ER, Barrett-Connor E, Orwoll ES. Antiepileptic drug use and rates of hip bone loss in older men: a prospective study. Neurology 2008;71:723–30.
[10] Kim SH, Lee JW, Choi KG, Chung HW, Lee HW. A 6-month longitudinal study of bone mineral density with antiepileptic drug monotherapy. Epilepsy Behav 2007;10:291–5.
[11] Sheth RD, Hermann BP. Bone mineral density with lamotrigine monotherapy for epilepsy. Pediatr Neurol 2007;37:250–4.
[12] Pack AM, Morrell MJ, Randall A, McMahon DJ, Shane E. Bone health in young women with epilepsy after one year of antiepileptic drug monotherapy. Neurology 2008;70:1586–93.
[13] Guo CY, Ronen GM, Atkinson SA. Long-term valproate and lamotrigine treatment may be a marker for reduced growth and bone mass in children with epilepsy. Epilepsia 2001;42:1141–7.
[14] Ensrud KE, Walczak TS, Blackwell T, Ensrud ER, Bowman PJ, Stone KL. Antiepileptic drug use increases rates of bone loss in older women: a prospective study. Neurology 2004;62:2051–7.

[15] Fekete S, Simko J, Gradosova I, Malakova J, Zivna H, Palicka V, et al. The effect of levetiracetam on rat bone mass, structure and metabolism. Epilepsy Res 2013. http://dx.doi.org/10.1016/j.eplepsyres.2013.08.012.
[16] Malakova J, Brozmanova H, Vorisek V, Prochazkova V, Palicka V. A capillary GC method using nitrogen phosphorus detection for determination of topiramate in patients with epilepsy. Chromatographia 2007;66(5/6):363–7.
[17] Gradosova I, Zivna H, Svejkovska K, Palicka V, Tichy M, Zivny P. The role of atorvastatin in bone metabolism in male albino Wistar rats. Pharmazie 2011;66:606–10.
[18] Ali II, Herial NA, Orris M, Horrigan T, Tietjen GE. Migraine prophylaxis with topiramate and bone health in women. Headache 2011;51:613–6.
[19] Vega D, Maalouf NM, Sakhaee K. Increased propensity for calcium phosphate kidney stones with topiramate use. Expert Opin Drug Saf 2007;6:547–57.
[20] Ahn H, Kim JM, Lee K, Kim H, Jeong D. Extracellular acidosis accelerates bone resorption by enhancing osteoclast survival, adhesion, and migration. Biochem Biophys Res Commun 2012;418:144–8.
[21] Bekastro V, Striano P, Gorgone G, Costa C, Ciampa C, Caccamo D, et al. Hyperhomocysteinemia in epileptic patients on new antiepileptic drugs. Epilepsia 2010;51(2):274–9.
[22] Linnebank M, Moskau S, Semmler A, Widman G, Stoffel-Wagner B, Weller M, et al. Antiepileptic drugs interact with folate and vitamin B12 serum levels. Ann Neurol 2011;69(2):352–9.
[23] Vacek TP, Kalani A, Voor MJ, Tyagi SC, Tyagi N. The role of homocysteine in bone remodeling. Clin Chem Lab Med 2013;51:579–90.
[24] Anderson GD. Pharmacogenetics and enzyme induction/inhibition properties of antiepileptic drugs. Neurology 2004;63:3–8.
[25] Ohta T, Wergedal JE, Matsuyama T, Baylink DJ, Lau KH. Phenytoin and fluoride act in concert to stimulate bone formation and to increase bone volume in adult male rats. Calcif Tissue Int 1995;56:390–7.
[26] Richard D, Picard F, Lemieux C, Lalonde J, Samson P, Deshaies Y. The effects of topiramate and sex hormones on energy balance of male and female rats. Int J Obes 2002;26(3):344–53.
[27] Merideth CH. A single-center, double-blind, placebo-controlled evaluation of lamotrigine in the treatment of obesity in adults. J Clin Psychiatry 2006 Feb;67(2):258–62.
[28] Daoud AS, Bataineh H, Otoom S, Abdul-Zahra E. The effect of Vigabatrin, Lamotrigine and Gabapentin on the fertility, weights, sex hormones and biochemical profiles of male rats. Neuro Endocrinol 2004;25(3):178–83.
[29] Nissen-Meyer LS, Svalheim S, Taubrll E, Reppe S, Lekva T, Solberg LB, et al. Levetiracetam, phenytoin, and valproate act differently on rat bone mass, structure, and metabolism. Epilepsia 2007;48(10):1850–60.
[30] Moro-Alvarez MJ, Díaz Curiel M, de la Piedra C, Mariñoso ML, Carrascal MT. Bone disease induced by phenytoin therapy: clinical and experimental study. Eur Neurol 2009;62(4):219–30.
[31] Onodera K, Takahashi A, Mayanagi H, Wakabayashi H, Kamei J, Shinoda H. Phenytoin-induced bone loss and its prevention with alfacalcidol or calcitriol in growing rats. Calcif Tissue Int 2001;69:109–16.
[32] Valimaki MJ, Tiihonen M, Laitinen K. Bone mineral density measured by dual-energy X-ray absorptiometry and novel markers of bone formation and resorption in patients on antiepileptic drugs. J Bone Miner Res 1994;9(5):631–7.
[33] Verrotti A, Greco R, Latini G, Morgese G, Chiarelli F. Increased bone turnover in prepubertal, pubertal, and postpubertal patients receiving carbamazepine. Epilepsia 2002;43(12):1488–92.
[34] Sato Y, Kondo I, Ishida S, Motooka H, Takayama K, Tomita Y, et al. Decreased bone mass and increased bone turnover with valproate therapy in adults with epilepsy. Neurology 2001;57(3):445–9.
[35] Verrotti A, Agostinelli S, Coppola G, Parisi P, Chiarelli F. A 12-month longitudinal study of calcium metabolism and bone turnover during valproate monotherapy. Eur J Neurol 2010;17(2):232–7.
[36] Zhang J, Wang KX, Wei Y, Xu MH, Su JM, Bao YG, et al. Effect of topiramate and carbamazepine on bone metabolism in children with epilepsy. Zhongguo Dang Dai Er Ke Za Zhj 2010;12(2):96–8.
[37] Forcelli PA, Kozlowski R, Snyder C, Kondratyev A, Gale K. Effects of neonatal antiepileptic drug exposure on cognitive, emotional, and motor function in adult rats. J Pharmacol Exp Ther Mar 2012;340(3):558–66.

11.3. Appendix 3

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The effect of lacosamide on bone tissue in orchidectomised male albino Wistar rats

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Aims. While most antiepileptic drugs (AEDs) have been associated with various adverse effects on bone health, for the recently introduced lacosamide (LCM) no corresponding data have been published. The present study evaluates the effect of LCM on bone mineral density, bone turnover markers, and bone mechanical strength in a rat model.
Methods. 16 orchidectomized Wistar rats were divided into control and experimental groups, 8 rats each. Dual energy X-ray absorptiometry was used to measure bone mineral density (BMD). As bone metabolism markers, the concentrations of bone markers were assayed in bone homogenate. In addition, both femurs were measured and used for biomechanical testing.
Results. Compared to the control group, we found lower BMD in the experimental group in the area of the left (8%) as well as the right femur (12%), all differences being statistically significant. In both femur diaphyses, but not in lumbar vertebrae, BMD was lower in the LCM group, suggesting a preferential effect on cortical bone. However, neither the thickness of the diaphyseal cortical bone nor the fragility in biomechanical testing was different between the groups. Of the bone metabolism markers, the significant decline was in procollagen type I N-terminal peptide (PINP) levels (37.4%), suggesting a decrease in osteoid synthesis.
Conclusion: We assume then that long-lasting exposure to LCM can represent a certain risk to the health of bone in the setting of gonadal insufficiency. Further studies will be needed to confirm these findings and to determine how high the risk will be in comparison to the other AEDs.

Key words: antiepileptic drugs, bone mineral density, bone turnover, biomechanical strength, lacosamide, PINP

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INTRODUCTION

Their use in epilepsy and the treatment of pain, and wide therapeutic use in psychiatry make antiepileptic drugs (AEDs) a pharmaco-epidemiologically important group of drugs both in the adult as well as the juvenile population[1-3]. Osteopathies occurring with long-term chronic antiepileptic treatment were first noted in the late sixties[4,5]. Since that time a number of theories have been proposed to explain why AEDs affect bone, but none explains all the reported effects. Most studies of the effects of AEDs on bone tissue are cross-sectional. There are only a few longitudinal studies, and there are limited data regarding the newer AEDs (ref.[6]).

Lacosamide (LCM) (SPM 927, formerly harkoseride), the R-enantiomer of 2-acetamido-*N*-benzyl-3-methoxypropionamide, is a chemical compound with anticonvulsant and anti-nociceptive properties. In November 2007, a new drug application was filed with the FDA for use of LCM as adjunctive therapy in the treatment of partial-onset seizures in adults with epilepsy. LCM was approved in Europe on September 3, 2008 as adjunctive therapy in the treatment of partial-onset seizures, with or without secondary generalization, for patients with epilepsy of 16 years or older[7]. Sex hormone deficiency increases the risk of developing antiepileptic drug-induced osteopathy (AEDs-O) (ref.[8]).

We report here our findings on the impact of LCM on bone mineral density (BMD), bone mineral content (BMC), bone metabolism markers, and bone biomechanical properties in orchidectomised (ORX) rats fed on LCM-enriched diet for 12 weeks. Apart from this study, to our knowledge there are no other full-text studies evaluating the effect of LCM on bone tissue. We have found one study referring to the absence of changes in BMD in juvenile dogs that has been published only in the form of an abstract[9].

METHODS

Animals

All animals received humane care in accordance with the guidelines set by the institutional Animal Use and Care Committee of Charles University in Prague, Faculty of Medicine in Hradec Kralove, Czech Republic. The protocol of the experiment was approved by the same committee. The experiments used eight-week-old male albino Wistar rats (Biotest s.r.o., Konarovice, Czech Republic). The animals were housed in groups of 4 in plastic cages. During the experimental period the animals were maintained under controlled conventional conditions (12 hours light and 12 hours dark, temperature 22±2 °C, air humidity 30-70%). Tap water, standard laboratory diet (SLD, VELAS, a.s., Lysa nad Labem, Czech Republic) and SLD enriched with LCM were given *ad libitum*. The weight of the rats was monitored once a week.

Experiment

Rats weighing (270±7 g) at the beginning of the experiment were divided into two groups of 8 animals:

1. CON-ORX: orchidectomised control fed with SLD
2. LCM-ORX: orchidectomised rat fed with SLD enriched with LCM (18 mg/25 g of the diet; Lacosamid, UCB Pharma)

At the beginning of the experiment the rats (CON-ORX and LCM-ORX) underwent bilateral orchidectomy under ether anaesthesia. On the second day after operation the LCM-ORX began to receive SLD enriched with LCM and the CON-ORX only SLD, both diets *ad libitum*. After 12 weeks, the animals were sacrificed by exsanguination from the abdominal aorta under ether anesthesia, and the obtained serum was aliquoted and stored at -80 °C for ensuing biochemical analyses. Both tibiae and femurs of the sacrificed rats were dissected free of soft tissue, wrapped in gauze moistened with saline and frozen to -80°C till the time of analysis.

Analysis of serum and bone homogenates

In the blood serum, the levels of osteoprotegerin (OPG) and insulin-like growth factor 1 (IGF-1) were determined by the ELISA (Enzyme-Linked Immunosorbent Assay) method. The blood serum levels of LCM were determined in the middle and at the end of the experiment. LCM was assayed by modified high-performance liquid chromatography with diode array detection[10]. Sample preparation included precipitation of plasma proteins: 200 µL of acetonitrile and 20 µL of zinc sulphate solution (10%) were added to 100 µL of plasma samples in 1.5-mL polypropylene centrifugation tubes. The tubes were vortexed for 120 seconds and centrifuged at 15,000 rpm for 10 minutes. The supernatant (30 µL) was injected into the HPLC system. Analysis was performed on a 2695 Waters Separations Module equipped with 996 photodiode array detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). Data acquisition and processing were provided with Empower Software (Waters). The analytical column was Zorbax SB-C8 (Agilent Technologies) – 150 x 4.6 mm, 3.5 µm. The analytical precolumn was Symmetry C18 Guard Column – 20 x 3.9 mm, 5 µm (Waters). The mobile phase was pumped at flow rate 0.8 mL/min and consisted of acetonitrile:formic acid 0.1 % (30:70, v/v). Temperature on the column was set at 30 °C, and injection volume was 30 µL. LCM concentration was determined at a wavelength of 215 nm (ref.[10].

In bone homogenate, the levels of the markers carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), aminoterminal propeptide of procollagen type I (PINP), bone alkaline phosphatase (BALP) and bone morphogenetic protein 2 (BMP-2) were determined also using the ELISA method. The homogenate was prepared from the tibia. After animal sacrifice, both tibiae were carefully excised; after removal of all the surrounding skin, muscle and other soft tissue, they were stored at -80 °C until required. The proximal part of the tibia (0.1 g) was disrupted and homogenized in 1.5 mL of phosphate buffer (PBS, PAA Laboratories GmbH, Pasching, Austria) with MagNA Lyser instrument (Roche Applied Science, Germany) at 6500 rpm for 20 s and cooled on the MagNA Lyser Cooling Block. This procedure was repeated three times.

The tissue homogenate was centrifuged at 10,000 g at 4 °C for 10 min. The supernatant was obtained and stored at -80 °C. Determination of the levels of bone markers was carried out using kits from Uscn Life Science Inc., Wuhan, China (PINP, Procollagen I N-Terminal Propeptide, pg/mL; OPG, Osteoprotegerin, pg/mL; IGF-1 Insulin Like Growth Factor 1, pg/mL; CTX-I, Cross Linked C-Telopeptide Of Type I Collagen; pg/mL; BALP, Alkaline Phosphatase, Liver/Bone/Kidney, ng/mL; BMP-2, Bone Morphogenetic Protein 2, pg/mL).

Dual energy X-ray absorptiometry analysis

The rat bone mineral density (BMD, g/cm^2) was measured by means of dual energy X-ray absorptiometry (DEXA) on a Hologic Delphi A device (Hologic, MA, USA) at the Osteocentre of the Faculty Hospital Hradec Kralove, Czech Republic. Before measurements, a tissue calibration scan was performed with the Hologic phantom for the small animal. Bone mineral density of the whole body, in the area of the lumbar vertebrae and in the area of the femur was evaluated by computer using the appropriate software programme for small animals (DEXA; QDR-4500A Elite; Hologic, Waltham, MA, USA). All animals were scanned by the same operator.

Biomechanical testing procedure

Mechanical testing of the rat femoral shaft and femoral neck was done with a special electromechanical custom-made testing machine (Martin Kosek & Pavel Trnecka, Hradec Kralove, Czech Republic) as described in a previous report[11]. For the three-point bending test, the femur was supported in the anteroposterior direction on a holding device, with the two support points 18 mm apart. A small stabilizing preload to 10 N was used to fix the bone between the contacts. A constant deformation rate of 6 mm/min as applied until maximal load failure and breaking strength (maximum load, N)

were recorded. Once the bone was broken, the thickness of the cortical part of the bone was measured by means of a sliding micrometer (OXFORD 0-25MM 30DEG POINTED MICROMETER, Victoria Works, Leicester, Great Britain). The proximal part of the femur was used for compression test of the femoral neck. The diaphysis of the bone was embedded into a container using a methacrylate resin and a vertical load was applied to the top of the femoral head. A small stabilizing preload to 10 N was applied and then advanced at a constant speed of 6 mm/min until failure of the femoral neck. The breaking strength (maximum load, N) was recorded by the measuring unit (Digitalanzeiger 9180, Burster praezisiosmesstechnik Gmbh & co kg, Gernsbach, Germany). All bones were analyzed by the same operator.

Analysis

Data were analyzed using Statistica v.10 (USA) software. Because the Shapiro–Wilk *W*-test for normality of data indicated that normal distribution was unlikely, non-parametric tests were used in subsequent analyses. Results were expressed in the form of medians, as well as lower and upper quartiles. Data of CON-ORX and LCM-ORX groups were compared using the Mann-Whitney U test to assess differences. Statistical significance was indicated by *P*-values <0.05 in all calculations.

RESULTS

Comparison of body composition showed a significantly lower fat mass in total (16.5 g) as well as in relative (4.1%) expression in those rats supplied with LCM. The contrast in fat expressed as a percentage was 18.3% ($100*(1-fat_{LCMR}/fat_{CONR})$ between the groups. Also, the weight of rats in the CON-ORX groups was found to be higher (37 g) compared to the LMC-ORX group, but this difference was not statistically significant – for details see Table 1.

Among the tested bone markers only aminoterminal propeptide of procollagen type I was significantly lower in the LCM-ORX group, by 29.6 pg/L, representing an intergroup contrast of 37.4%. Particular values and a detailed list of the other followed parameters are displayed in Table 2.

The mineral density of bone evaluated in the whole body and in the area of the lumbar vertebrae did not show any significant differences between the groups; however in the area of the left as well as the right femur we found significantly lower density (left 0.18 g/cm^2 and right 0.17 g/cm^2) in the LCM-ORX group compared to the control group (left BMD 0.20 g/cm^2 and right BMD 0.20 g/cm^2). The contrast between groups was 8.8% in the left BMD and 12.0% in the right BMD. The mineral content did not differ statistically between the groups. The listed values are medians; for interquartile intervals and other significant parameters see Table 3.

Table 1. Body weight and fat mass.

Parameter	CON-ORX (n=8) [25% - 75%]	LCM-ORX (n=8) [25% - 75%]	Man-Whitney U Test [*P*]
Weight (g)	523 [489 - 543]	486 [471 - 499]	0.189
Fat (g)	**90.3 [83.1 – 127.2]**	**73.8 [65.1 – 85.2]**	**0.024**
Fat (%)	**22.1 [20.1 – 26.7]**	**18.0 [16.1 – 20.0]**	**0.041**
Lean body mass (g)	326.2 (310.9 – 378.8)	340.9 (326.9 – 350.9)	0.495

Data are expressed as median (25th – 75th percentiles)

Table 2. Levels of bone markers.

Parameter	CON-ORX (n=8) [25% - 75%]	LCM-ORX (n=8) [25% - 75%]	Man-Whitney U Test [*P*]
OPG (pg/mL)	54.7 [51.9 – 58.5]	57.9 [51.1 – 54.5]	0.372
IGF-1 (pg/mL)	1.11 [1.06 – 1.21]	1.17 [1.05 – 1.29]	0.564
CTX-1 (pg/mL)	0.97 [0.76 – 1.57]	0.90 [0.64 – 1.63]	0.772
PINP (pg/mL)	**79.0 [71.9 – 81.5]**	**49.4 [34.5 – 67.6]**	**0.005**
BALP (ng/mL)	1.16 [0.76 – 1.46]	0.88 [0.70 – 1.14]	0.495
BMP-2 (pg/mL)	876 [610 - 1010]	824 [763 - 1055]	0.958

OPG, osteoprotegerin; IGF-1, insulin-like growth factor 1; CTX-I, carboxy-terminal cross-linking telopeptide of type I collagen; PINP, aminoterminal propeptide of procollagen type I; BALP, bone alkaline phosphatase; BMP-2, bone morphogenetic protein 2.
Data are expressed as median (25th – 75th percentiles)

Table 3. Comparison of values from DXA analysis and values of biomechanical testing.

	CON-ORX (n=8) [25% - 75%]	LCM-ORX (n=8) [25% - 75%]	Man-Whitney U Test [*P*]
DEXA			
Whole body BMD, g/cm^2	0.175 [0.171 – 0.176]	0.169 [0.165 – 0.171]	0.066
W BMC (g)	14.0 [13.3 – 14.,4]	13.5 [13.1 – 13.9]	0.318
LF BMD, g/cm^2	**0.197 [0.192 – 0.199]**	**0.179 [0.169 – 0.185]**	**0.004**
LF BMC (g)	0.276 [0.258 – 0.290]	0.266 [0.259 – 0.279]	0.793
RF BMD, g/cm^2	**0.198 [0.188 – 0.211]**	**0.175 [0.160 – 0.179]**	**0.001**
RF BMC (g)	0.259 [0.236 – 0.280]	0.259 [0.248 – 0.284]	0.564
Lumbar columna (L3-L5) BMD (g/cm^2)	0.214 [0.210 – 0.223]	0.212 [0.207 – 0.223]	0.793
Lumbar columna (L3-L5) BMC (g)	0.571 [0.546 – 0.598]	0.575 [0.542 – 0.614]	0.495
Biomechanical testing measurement			
LF length, mm	37.5 [36.6 – 38.0]	37.3 [36.9 – 38.6]	0.495
RF length, mm	37.4 [36.6 – 37.8]	37.4 [37.2 – 37.8]	0.958
LF diameter, mm	3.63 [3.51 – 3.95]	3.64 [3.62 – 3.,67]	0.958
RF diameter, mm	3.63 [3.53 – 3.72]	3.65 [3.47 – 3.95]	0.753
Cortical LF thickness, mm	0.720 [0.665 - 0.740]	0.705 [0.695 – 0.750]	0.263
Cortical RF thickness, mm	0.705 [0.665 - 0.735]	0.695 [0.680 – 0.740]	0.958
Maximal load of the left femoral shaft, N	214 [197 - 236]	218 [207 - 229]	1.000
Maximal load of the right femoral shaft, N	216 [203 - 230]	224 [204 - 235]	0.564
Maximal load of left femoral neck, N	144 [135 - 152]	146 [128 - 163]	0.897
Maximal load of right femoral neck, N	160 [138 - 178]	149 [141 - 161]	0.355

LF- left femur, RF – right femur, BMD – bone mineral density, BMC – bone mineral content. Data are expressed as median (25th – 75th percentiles) CON-ORX, orchidectomised control fed with standard laboratory diet; LCM-ORX, orchidectomised rat fed with standard laboratory diet enriched with lacosamide.

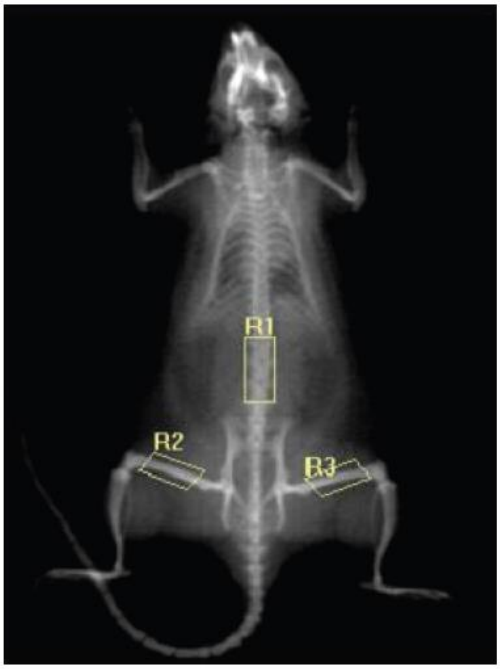

Fig. 1. Evaluation of BMD in three areas of the rat skeleton.
R1 – lumbar columna (L3-L5);
R2 – left femur;
R3 – right femur

The Mann-Whitney U test also showed that medians of biomechanical and geometric parameters of right and left femurs did not differ (see Table 3).

The level of lacosamide in LCM-ORX at the end of the experiment: 13.49 umol/L (12.96 - 14.59).

DISCUSSION

The aim of this study was to evaluate the effect of LCM on BMD, bone markers, and biomechanical quality using the mature ORX rat model. The effect of LCM on bone tissue has not yet been investigated, except for studies Cornet et al 2010 in juvenile dogs (published only in the form of an abstract) (ref.[9]). In this study, ORX rats fed with SLD enriched with LCM (LCM-ORX) had significant loss of BMD at the left and right femur after 12 weeks when compared to the control (CON-ORX). However, no significant differences in biomechanical and geometric parameters of rat right and left femurs were observed. Evaluation of bone turnover using biochemical markers specific for both bone formation (BALP, BMP-2, PINP, OPG, IGF-1) and bone resorption (CTX-I) was without significant difference with the exception of PINP.

With respect to AEDs, phenytoin (PHT), phenobarbital (PB), and primidone (PRM) are most consistently associated with a negative impact on bone. Carbamazepine (CBZ) and valproate (VPA) may also result in bone abnormalities, but the data are mixed. Current studies suggest that lamotrigine (LTG) has limited (if any) effect,

but again, the data are inconsistent. Other AEDs have received limited study[12] but there is increasing evidence supporting the notion that topiramate (TPM) may have negative impact on bone health[13-15].

The significance of bone turnover markers for diagnosis of antiepileptic drug-induced osteopathy (AEDs-O) is controversial: although PHT is well-known to cause significant loss of BMD and BMC, only modest changes in markers of bone turnover have been observed in animals[16,17]. Similarly, in a longitudinal study of premenopausal women treated with PHT, bone turnover markers remained unchanged after 1 year, except for a significant decline in urine N-telopeptide. This result is unclear and difficult to explain, particularly in view of the significant observed femoral neck bone loss[18]. Conflicting data exist regarding the effects of CBZ on BMD and bone turnover[8,19-21]. VPA in animals reduced BMD and BMC and increased bone turnover[16]. There are mixed data in humans. Some have observed that VPA monotherapy decreased BMD and increased both markers of bone formation and resorption significantly[22,23], but in the longitudinal study of young women mentioned above, the BMD was stable and bone turnover markers remained unchanged after 1 year of VPA treatment[18]. There are scarce data regarding LTG. So far LTG has not been shown to cause significant effects on BMD and bone turnover[18,21], except significantly increased osteocalcin, a marker of bone formation referred to in one study[24].

In our study we noticed a significant change in the PINP. PINP is a marker of bone formation, which was significantly lower in LCM-ORX. To our knowledge, there have appeared no studies with AEDs (or with CYP2C19 inhibitors) in which changes in the level of PINP were monitored. Some further research will be necessary to verify the role and the importance of PINP in the diagnosis, and more precisely in the pathophysiology of AEDs-O. We discovered only one prospective study testing the influence of LCM on BMD in gonadally intact subjects, in which the authors claim an absence of influence of LCM on BMD (ref.[9]). However, we monitored the significant decline of BMD at the left and right femur. We assume then that long-lasting exposure to LCM can represent a certain risk to the health of bone in the setting of the gonadal insufficiency. It is complicated to determine how high the risk will be in comparison to the other AEDs. In the case of levetiracetam (LEV) we monitored a significant decline of BMD on the same model in only 1 out of 4 locations of measurement (left femur), and moreover, we did not record significant changes in the mechanical durability of the bone[25]. In the case of LTG and TPM, we recorded on the same model both significant decline of BMD (at 3 measurement locations out of 4) and significant reduction in the mechanical durability of the bone (supplied data). The old AEDs have not been tested on the ORX rat model so far. However, in models of gonadally intact rats, significant reduction of BMD after using the old AEDs has been observed (see text above).

The mechanism of the effect of LCM on bone is unclear. LCM has been shown to produce a significant effect in rodents consistent with anxiolysis: LCM increased the suppression ratio in a conditioned emotional response test, and reduced the number of marbles buried in the marble burying assay[26]. In rodents, physical activity prevents decrease in BMD as it does in humans, which suggests that increased physical activity could be useful in the prevention of bone mineral loss, regardless of gonadal hormone deficiency[27]. Therefore reduced locomotor activity could be the factor contributing to significant decrease LF-BMD and RF-BMD in LCM-ORX compared to CON-ORX.

In our study, LCM-ORX had significantly lower fat mass compared with CON-ORX. LCM showed no potential to induce or to inhibit cytochrome P450 isoforms except for CYP2C19 (60% inhibition) (ref.[28]). CYP2C19 is one of the (most) important isoforms involved in the metabolism of sex hormones. CYP2C19 catalyzes the 17β-hydroxy dehydrogenation and 16α hydroxylation of estradiol, and 17β-hydroxy dehydrogenation is the main metabolism pathway at low estradiol concentrations[29,30]. The main pathway of testosterone oxidative metabolism by human liver microsomes is the formation of 1β-, 2α-/β-, 6β-, 15β-, and 16β-hydroxytestosterones, mainly catalyzed by CYP2C9, CYP2C19, and CYP3A4 (ref.[31]). In a study in the ORX rat model with different doses of testosterone replacement there were no significant differences in fat mass[32], and in another study, 17β-estradiol prevented the ORX-related increase of fat mass, whereas 5α-dihydrotestosterone did not[33]. Thus we propose that inhibition of estrogen metabolism may be the cause of lower fat mass in LCM-ORX rather than inhibition of androgen metabolism.

There are several limitations that should be considered in evaluating the present study. Firstly, the three-month follow-up period may be too short for monitoring changes in biomechanical properties of the bone tissue. Secondly, the sample size was small: although statistical significance is evident, the capacity to identify possible variables of confusion is limited. Finally, behavioral activity was neither controlled nor assessed.

CONCLUSION

In summary, in this study, LCM-ORX had significant loss of BMD at the left and right femur and significant decline in PINP compared to the control. It will be necessary to carry out further studies to validate the findings of this study and to elucidate the exact mechanism of the significant loss of femur BMD. Further studies are warranted to establish whether LCM has a clinically significant effect on BMD exclusively in the model of gonadectomized rats, or whether the effect applies also in the model of gonadally intact animals, and in the respective human models.

Despite the above-mentioned limitations, this study contributes significantly to our knowledge about the effect of LCM on bone tissue.

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CONFLICT OF INTEREST STATEMENT

Author's conflict of interest disclosure: None declared.

REFERENCES

1. Savica R, Beghi E, Mazzaglia G, Innocenti F, Brignoli O, Cricelli C, Caputi AP, Musolino R, Spina E, Trifirń G. Prescribing patterns of antiepileptic drugs in Italy: nationwide population-based study in the years 2000-2005. Eur J Neurol 2007;14(12):1317-21.
2. Johannessen LC, Larsson PG, Rytter E, Johannessen SI. Antiepileptic drugs in epilepsy and other disorders-A population-based study of prescriptions. Epilepsy Res 2009;87(1):31-9.
3. Van de Vrie-Hoekstra NW, de Vries TW, van den Berg PB, Brouwer OF, de Jong-van den Berg LT. Antiepileptic drug utilization in children from 1997-2005 a study from the Netherlands. Eur J Clin Pharmacol 2008;64(10):1013-20.
4. Schmid F. Osteopathien bei antiepileptischer D auerbehandlung. Fortschr Med 1967;85:381-2.
5. Kruse R. Osteopathien bei antioepileptischer Langzeittherapie (vorläufige Mitteilung). Mschr Kinderheilkd 1968;116:378-80.
6. Pack A. Bone health in people with epilepsy: is it impaired and what are the risk factors? Seizure 2008;17(2):181-6.
7. Halford JJ, Lapointe M. Clinical Perspectives on Lacosamide. Epilepsy Currents 2009;9(1):1-9.
8. Carbone LD, Johnson KC, Robbins J, Larson JC, Curb JD, Watson K, Gass M, Lacroix AZ. Antiepileptic drug use, falls, fractures, and BMD in postmenopausal women: findings from the women's healthinitiative (WHI). J Bone Mineral res 2010;25(4):873-81.
9. M. Cornet, D. Tytgat and M. Léonard. Lacosamide does not alter bone densitometry parameters in juvenile dogs. Abst. American Epilepsy Society 2010; Abstract No.2.191.
10. Greenaway C, Ratnaraj N, Sander JW, Patsalos PN. A High-performance liquid chromatography assay to monitor the new antiepileptic drug lacosamide in patients with epilepsy. Ther Drug Monit 2010;32(4):448-52.
11. Gradosova I, Zivna H, Svejkovska K, Palicka V, Tichy M, Zivny P. The role of atorvastatin in bone metabolism in male albino Wistar rats. Pharmazie 2011;66:606-10.
12. Pack AM. Treatment of epilepsy to optimize bone health. Curr Treat Options Neurol 2011;13(4):346-54.
13. Coppola G, Fortunato D, Auricchio G, Mainolfi C, Operto FF, Signoriello G, Pascotto A, Salvatore M. Bone mineral density in children, adolescents, and young adults with epilepsy. Epilepsia 2009;50(9):2140-6.
14. Heo K, Rhee Y, Lee HW, Lee SA, Shin DJ, Kim WJ, Song HK, Song K, Lee BI. The effect of topiramate on bone mineral density and markers of bone mineral metabolism in premenopausal women with epilepsy. Epilepsia 2011;52(10):1884-9.
15. Ali II, Herial NA, Orris M, Horrigan T, Tietjen GE. Migraine prophylaxis with topiramate and bone health in women. Headache 2011;51(4):613-6.
16. Nissen-Meyer LS, Svalheim S, Taubøll E, Reppe S, Lekva T, Solberg LB, Melhus G, Reinholt FP, Gjerstad L, Jemtland R. Levetiracetam, phenytoin, and valproate act differently on rat bone mass, structure, and metabolism. Epilepsia 2007;48(10):1850-60.
17. Moro-Alvarez MJ, Díaz Curiel M, de la Piedra C, Mariñoso ML, Carrascal MT. Bone disease induced by phenytoin therapy: clinical and experimental study. Eur Neurol 2009;62(4):219-30.
18. Pack AM, Morrell MJ, Randall A, McMahon DJ, Shane E. Bone health in young women with epilepsy after one year of antiepileptic drug monotherapy. Neurology 2008;70(18):1586-93.
19. Valimaki MJ, Tiihonen M, Laitinen K. Bone mineral density measured by dual-energy x-ray absorptiometry and novel markers of bone formation and resorption in patients on antiepileptic drugs. J Bone Miner Res 1974;9(5):631-7.
20. Verrotti A, Greco R, Latini G, Morgese G, Chiarelli F. Increased bone turnover in prepubertal, pubertal, and postpubertal patients receiving carbamazepine. Epilepsia 2002;43(12):1488-92.
21. Sheth RD, Hermann BP. Bone mineral density with lamotrigine monotherapy for epilepsy. Pediatr Neurol 2007;37(4):250-4.
22. Sato Y, Kondo I, Ishida S, Motooka H, Takayama K, Tomita Y, Maeda H, Satoh K. Decreased bone mass and increased bone turnover with valproate therapy in adults with epilepsy. Neurology 2001;57(3):445-9.
23. Verrotti A, Agostinelli S, Coppola G, Parisi P, Chiarelli F. A 12-month longitudinal study of calcium metabolism and bone turnover during valproate monotherapy. Eur J Neurol 2010;17(2):232-7.
24. Kim SH, Lee JW, Choi KG, Chung HW, Lee HW. A 6-month longitudinal study of bone mineral density with antiepileptic drug monotherapy. Epilepsy Behav 2007;10(2):291-5.
25. Fekete S, Simko J, Gradosova I, Malakova J, Zivna H, Palicka V, Zivny P. The effect of levetiracetam on rat bone mass, structure and metabolism. Epilepsy research 2013;107(1-2):56-60.
26. Higgins GA, Breysse N, Undzys E, Kuo C, Joharchi N, Derksen DR, Xin T, Isaac M, Slassi M. The anti-epileptic drug lacosamide (Vimpat) has anxiolytic property in rodents. Eur J Pharmacol 2009;624(1-3):1-9.
27. Horcajada-Molteni MN, Davicco MJ, Coxam V, Lebecque P, Dominguez B, Ritz P, Culioli J, Barlet JP. Treadmill running starting 3 months after orchidectomy restores femoral bone mass in rats. Eur J Appl Physiol Occup Physiol 1999;79(3):251-9.
28. Beyreuther BK, Freitag J, Heers C, Krebsfänger N, Scharfenecker U, Stöhr T. Lacosamide: A Review of Preclinical Properties. CNS Drug Rev 2007;13(1):21-42.
29. Cribb AE, Knight MJ, Dryer D, Guernsey J, Hender K, Tesch M. Role of polymorphic human cytochrome P450 enzymes in estrone oxidation. Cancer Epidemiol Biomarkers Prev 2006;15:551-8.
30. Cheng ZN, Shu Y, Liu ZQ, Wang LS, Ou-Yang DS, Zhou HH. Role of cytochrome P450 in estradiol metabolism in vitro. Acta Pharmacol Sin 2001;22:148-54.
31. Man Ho Choi, Paul L. Skipper, John S. Wishnok, Steven R. Tannenbaum: Characterization of testosterone 11β-hydroxylation catalyzed by human liver microsomal cytochromes P450. Drug metabolism and disposition 2005;33:714-8.
32. Vanderschueren D, Vandenput L, Boonen S, Van Herck E, Swinnen JV, Bouillon R. An aged rat model of partial androgen deficiency: prevention of both loss of bone and lean body mass by low-dose androgen replacement. Endocrinology 2000;141(5):1642-7.
33. Vandenput L, Boonen S, Van Herck E, Swinnen JV, Bouillon R, Vanderschueren D. Evidence from the aged orchidectomized male rat model that 17beta-estradiol is a more effective bone-sparing and anabolic agent than 5alpha-dihydrotestosterone. J Bone Miner Res 2002;7(11):2080-6.

11.4. Appendix 4

Original Paper

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Effect of Mirtazapine on Rat Bone Tissue after Orchidectomy

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Key Words
Mirtazapine · Bone mineral density · Bone markers · Biomechanical properties

Abstract
Objective: Our study aimed to investigate the effect of mirtazapine on bone metabolism in the orchidectomized rat model. ***Methods:*** Rats were divided into three groups. A sham-operated control group (SHAM group) and a control group after orchidectomy (ORX group) received the standard laboratory diet (SLD). An experimental group after orchidectomy (ORX MIRTA group) received SLD enriched with mirtazapine for 12 weeks. Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry. Bone marker concentrations of osteoprotegerin (OPG), amino-terminal propeptide of procollagen type I, bone alkaline phosphatase (BALP), sclerostin and bone morphogenetic protein 2 were examined in bone homogenate. The femurs were used for biomechanical testing. ***Results:*** Compared with the control ORX group, we found a lower BMD in the ORX MIRTA group. The differences were statistically significant, although not in the lumbar vertebrae. BMD was lower in the MIRTA group, suggesting a preferential effect on cortical bone. However, although the thickness of the diaphyseal cortical bone was not different, the fragility in the femoral neck area was statistically significantly different between the groups in biomechanical testing. Regarding the bone metabolism markers, there was a significant decrease in OPG and BALP levels, suggesting a reduction in osteoid synthesis. ***Conclusions:*** The results suggest that prolonged use of mirtazapine may have a negative effect on the synthesis of bone and on its mechanical strength, especially in the femoral neck. Further studies are warranted to establish whether mirtazapine may have a clinically significant adverse effect on bone exclusively in the model of gonadectomized rats, or whether the effect occurs also in the model of gonadally intact animals and in respective human models.

Introduction

Several categories of drugs that act on the central nervous system, such as antidepressants, anticonvulsants and opioids, have been shown to increase the risk of fracture and/or osteoporosis [1, 2]. On the other hand, the use of these drugs may be a marker of factors such as poor health, medical conditions and lifestyle behaviors that are associated with a higher risk of fracture and/or bone loss. Nearly all previous investigations into the use of these drugs and the risk of fracture or/and bone loss have been

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cross-sectional studies with inadequate control of confounders. Hence, there is a strong need for prospective studies to prove a causal link between medication and adverse effects on bone.

Evidence from psychiatric morbidity surveys suggests that the current level of major depression in the general population is about 5% but is higher in the older age groups; antidepressants are thus one of the most commonly prescribed drugs, particularly to people aged ≥65 years [3].

Mirtazapine, i.e. 2-methyl-hexahydropyrazino[2,1-a]pyrido[2,3-c][2]benzazepine, is the only representative of the noradrenergic and specific serotonergic antidepressant class. It is a novel antidepressant which has a unique dual mode of action. Mirtazapine affects norepinephrine transmission via blockade of central α_2-adrenoceptors and is a potent serotonin (5-HT_2 and 5-HT_3) antagonist, thereby increasing serotonergic stimulation via the 5-HT_1 receptor. It has no significant affinity for dopamine receptors, a low affinity for muscarinic cholinergic receptors and no effect on monoamine reuptake [4]. The mechanism of action of antidepressants in the regulation of bone tissue is not fully understood. A recent study found that transporters of serotonin may play a role in bone metabolism, and that medications that affect these transporter systems may also affect bone metabolism [5].

We here report the impact of mirtazapine on bone mineral density (BMD), bone mineral content (BMC), bone metabolism markers and biomechanical properties of bone. We used the orchidectomized rat model (the model for androgen-deficient osteoporosis), in which a higher sensitivity can be expected for the detection of a possible risk of xenobiotics for bone health.

Methods

Animals

All animals received humane care in accordance with the guidelines set by the Institutional Animal Use and Care Committee of the Faculty of Medicine in Hradec Kralove, Charles University in Prague (Hradec Kralove, Czech Republic). The protocol of the experiment was approved by the same committee. In the experiments, 8-week-old male albino Wistar rats were used (Biotest s.r.o., Konarovice, Czech Republic). The animals were hosted in groups of 4 in plastic cages. During the experimental period, they were maintained under controlled conventional conditions (12 h light and 12 h dark, temperature 22 ± 2°C, air humidity 30–70%). Tap water and standard laboratory diet (SLD; VELAS a.s., Lysa nad Labem, Czech Republic) or SLD enriched with mirtazapine were given ad libitum. The weight of the rats was monitored once a week.

Experimental Design

Rats weighing 200 ± 10 g at the beginning of the experiment were divided into three groups of 8 animals: (1) sham-operated controls fed with SLD (SHAM); (2) orchidectomized controls fed with SLD (ORX), and (3) orchidectomized rats fed with SLD enriched with mirtazapine (ORX MIRTA; 1.98 mg/25 g of diet; Mirtazapin Krka, Czech Republic).

At the beginning of the experiment, the ORX and ORX MIRTA rats underwent bilateral orchidectomy under ether anesthesia; the SHAM rats underwent only scrotal incision. From the second day after the operation, the ORX MIRTA group received SLD enriched with mirtazapine, and the SHAM and ORX groups only SLD, both diets ad libitum. After 12 weeks, the animals were sacrificed by exsanguination from the abdominal aorta under ether anesthesia, and the obtained serum was aliquoted and stored at –80°C for the ensuing biochemical analyses. After sacrifice of the rats, both tibiae and femurs were dissected free of soft tissue, wrapped in gauze moistened with saline and frozen to –80°C till required for analysis.

Analysis of Serum and Bone Homogenates

Serum levels of mirtazapine were determined using the HPLC-MS system. Sample preparation included precipitation of plasma proteins: 500 µl of 40 mmol/l zinc sulfate in 66% methanol was added to a polypropylene tube containing 500 µl of plasma sample and 50 µl of 2,000 ng/ml reserpine as the internal standard according to the method described in the report by Borges et al. [6].

Bone homogenate was prepared from the tibiae. The proximal part of the bone (0.1 g) was disrupted and homogenized in 1.5 ml of phosphate-buffered saline (Penta, Prague, Czech Republic) with a MagNA Lyser instrument (Roche Applied Science, Germany) at 6,500 rpm for 20 s and cooled on the MagNA Lyser Cooling Block. This procedure was repeated 3 times. The raw tissue homogenate was centrifuged at 10,000 *g* at 4°C for 10 min, and the resulting supernatant was collected and stored at –80°C.

Levels of the markers osteoprotegerin (OPG), amino-terminal propeptide of procollagen type I (P1NP), bone alkaline phosphatase (BALP), sclerostin and bone morphogenetic protein 2 (BMP-2) were determined in this bone homogenate, also using the ELISA method. Bone marker levels were determined using ELISA kits from USCN Life Science Inc. (Wuhan, China; P1NP in pg/ml; OPG in pg/ml; BALP in ng/ml; BMP-2 in pg/ml; sclerostin in ng/ml).

Dual-Energy X-Ray Absorptiometry Analysis

BMD (in g/cm^2) was measured by means of dual-energy X-ray absorptiometry (DXA) on a Hologic Delphi A device (Hologic, Waltham, Mass., USA) at the Osteocentre of the Faculty Hospital in Hradec Kralove. Before measurements, a tissue calibration scan was performed with the Hologic phantom for small animals. BMD of the whole body, in the area of the lumbar vertebrae and in the area of the femur were evaluated by computer using the appropriate software program for small animals (DXA; QDR-4500A Elite; Hologic) (fig. 1). All animals were scanned by the same operator.

Biomechanical Testing Procedure

Mechanical testing of the rat femoral shafts and femoral necks was done with a special electromechanical custom-made testing machine (Martin Kosek & Pavel Trnecka, Hradec Kralove, Czech Republic) according to the method described in our previous re-

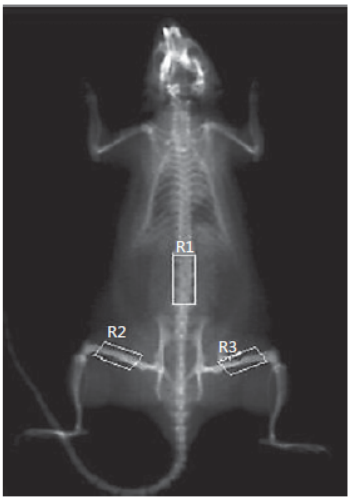

Fig. 1. Evaluation of BMD in three areas of the rat skeleton. R1 = Lumbar columna (L3–L5); R2 = left femur; R3 = right femur.

port [7]. For the 3-point bending test, the femur was placed on a holding device with the 2 support points 18 mm apart. A small stabilizing preload to 10 N was applied in the anteroposterior direction to fix the bone between the contacts. A constant deformation rate of 6 mm/min was generated until maximal load failure and breaking strength (maximum load, N) had been recorded. After the bone was broken, the thickness of the cortical part of the bone was measured by means of a sliding micrometer (OXFORD 0–25 mm 30 deg Pointed Micrometer; Victoria Works, Leicester, UK). The proximal part of the femur was used for compression testing of the femoral neck. The diaphysis of the bone was embedded into a container using a methacrylate resin, and a vertical load was applied to the top of the femoral head. A small stabilizing preload to 10 N was applied and increased at a constant speed of 6 mm/min until failure of the femoral neck. The breaking strength (maximum load, N) was recorded with the measuring unit (Digitalanzeiger 9180; Burster Präzisionsmesstechnik GmbH & Co KG, Gernsbach, Germany). All bones were analyzed by the same operator.

Statistical Analysis

Statistical analysis was performed using the program NCSS 2007 (Number Cruncher Statistical System; NCSS, Kaysville, Utah, USA). Comparisons of the parameters under study were made by analysis of variance with post hoc multiple comparison with Fisher's LSD test and by Kruskal-Wallis nonparametric analysis of variance with post hoc multiple comparison with Dunn's test (and Bonferroni modifications). Differences were considered significant at $p < 0.05$. The results are derived from measurements made 12 weeks after the experiment and are presented as medians (25th–75th percentiles).

Results

The level of mirtazapine in the ORX MIRTA group at the end of the experiment was 60 µg/l (45–60 µg/l).

Body Weight and Composition and Tissue Weight

Orchidectomy caused an increase in weight in the SHAM group in comparison with the ORX control group. The weight of the experimental ORX MIRTA group decreased, but this was statistically nonsignificant versus the control group. DXA revealed that the experimental group showed an increase in fat mass versus the control group. There were no significant differences in lean body mass between the experimental and the control group (table 1).

Levels of Bone Markers

Bone markers from specimens of the proximal tibia were measured to assess the effects of orchidectomy and treatment on bone formation. Determination of the levels of OPG revealed their decrease in the ORX versus the SHAM group. Levels of sclerostin were significantly increased. In the ORX control group, the levels of OPG, BALP, P1NP and BMP-2 were decreased versus the ORX MIRTA group, but those of P1NP and BMP-2 not significantly so. The results from the ELISA are shown in table 2.

Dual-Energy X-Ray Absorptiometry

In the ORX group, a significant decrease in BMD and BMC of the whole body and also in the area of the lumbar vertebrae and both femurs was demonstrated compared with the SHAM group. In the ORX MIRTA group there was a significant decrease in BMD and BMC of the whole body and both femurs, but the BMD and BMC of the lumbar vertebrae were unchanged versus the ORX control group. The results are shown in table 3.

Biomechanical Testing

In the SHAM group there was a statistically significant increase in the length of both femurs, and an increase in diameter which was not statistically significant, compared with the ORX control group. After mirtazapine administration there was a statistically significant decrease in length, and a decrease in thickness (not statistically significant), of the cortical bone as compared with the ORX group. Testing of the mechanical strength of the bone tissue by means of 3-point bending revealed a statistically significant decrease in maximal load values in the ORX versus the SHAM group. After mirtazapine administration there was a statistically significant decrease in maxi-

Table 1. Body weight and fat mass at the end of the experiment

	SHAM group	ORX group	p value
Weight, g	562 (541–622)	474 (460.25–490)*	*0.0018*
Fat, %	19.6 (16.95–20.85)	23 (20.825–28.875)*	*0.0171*
Lean body mass, g	368.7 (359.85–399.35)	285.4 (278.6–303.3)*	*0.0003*
	ORX group	ORX MIRTA group	p value
Weight, g	474 (460.25–490)	452 (431.25–486.25)	0.2337
Fat, %	23 (20.825–28.875)	26.25 (24.275–29.275)	0.7265
Lean body mass, g	285.4 (278.6–303.3)	265.95 (261.475–289.775)	0.2827

Data are expressed as medians (25th–75th percentiles). * $p < 0.05$ (significant values in italics).

Table 2. Levels of bone markers

	SHAM group	ORX group	p value
OPG, pg/ml	2.23 (2.12–2.46)	1.92 (1.56–2.08)*	*0.0111*
Sclerostin, ng/ml	0.268 (0.221–0.279)	0.344 (0.288–0.381)*	*0.0085*
P1NP, pg/ml	217.21 (164.71–248.82)	201.92 (182.58–235.28)	0.9015
BALP, ng/ml	20.09 (17.21–20.51)	18.31 (17.04–22.30)	0.7539
BMP-2, pg/ml	25.14 (21.78–26.65)	24.88 (22.42–27.57)	0.7502
	ORX group	ORX MIRTA group	p value
OPG, pg/ml	1.92 (1.56–2.08)	1.44 (1.32–1.55)*	*0.0181*
Sclerostin, ng/ml	0.344 (0.288–0.381)	0.377 (0.328–0.428)*	*0.0397*
P1NP, pg/ml	201.92 (182.58–235.28)	170.13 (152.15–200.91)	0.1589
BALP, ng/ml	18.31 (17.04–22.30)	9.01(7.91–11.81)*	*0.0005*
BMP-2, pg/ml	24.88 (22.42–27.57)	19.90 (18.64–24.96)	0.2928

Data are expressed as medians (25th–75th percentiles). * $p < 0.05$ (significant values in italics).

mal load of the femoral neck, but the maximal load of the femoral shaft was unchanged versus the ORX group (table 3.)

Discussion

Mirtazapine is a commonly prescribed medication in the treatment of depressive disorder. The effect of mirtazapine on bone metabolism has not yet been sufficiently investigated, and the aim of this study was to do so. To our knowledge, this study is the first clinical investigation to examine the effects of mirtazapine on bone metabolism in rats.

At the end of the experiment, the level of mirtazapine in the ORX MIRTA group was 60 µg/l (45–60 µg/l), which is equivalent to therapeutic levels of the drug (30–80 µg/l). In rats 12 weeks after orchidectomy, we found a significant decrease in body weight, BMD and lean body mass as well as an increase in fat mass. These results confirm the findings from previous studies in rats, i.e. that a deficiency of androgens negatively affects body composition, and establishes these animals as suitable models for investigating the androgenic modulation of body composition [8, 9]. In the ORX MIRTA group, these changes were not demonstrated in comparison with the ORX group.

The mechanism of the effect of mirtazapine on bone is unclear. Mirtazapine acts via the 5-HT transporter and also inhibits the norepinephrine transporter. These drugs have an alerting effect, impairing sleep duration and quality, thus causing insomnia, which may result in daytime drowsiness. This can contribute to increasing the risk of

Table 3. Comparison of values from DXA analysis and values from biomechanical testing

	SHAM group	ORX group	p value
Densitometry (BMD)			
Whole body, g/cm^2	0.184 (0.181–0.185)	0.164 (0.161–0.167)*	*0.00001*
Left femur, g/cm^2	0.194 (0.191–0.206)	0.176 (0.165–0.182)*	*0.00589*
Right femur, g/cm^2	0.199 (0.197–0.214)	0.181 (0.167–0.184)*	*0.00487*
Lumbar vertebrae (L3–L5), g/cm^2	0.248 (0.234–0.251)	0.203 (0.193–0.213)*	*0.0002*
Biomechanical testing			
Left femur length, mm	39 (38.27–39.24)	37.22 (36.88–37.67)*	*0.0007*
Right femur length, mm	39.02 (38.44–39.25)	37.31 (36.58–37.96)*	*0.0027*
Left femur diameter, mm	3.77 (3.64–3.83)	3.65 (3.5–3.69)	0.3120
Right femur diameter, mm	3.79 (3.72–3.9)	3.71 (3.58–3.88)	0.5028
Maximal load of the left femoral shaft, N	227 (224–236.5)	199 (191–217)*	*0.0269*
Maximal load of the right femoral shaft, N	217.4 (198–225)	183 (179.25–191.25)*	*0.0299*
Maximal load of left femoral neck, N	171 (139.5–184)	150.5 (130–165)*	*0.0484*
Maximal load of right femoral neck, N	160 (143–181.5)	152.43 (136–166)*	*0.0489*
	ORX group	ORX MIRTA group	p value
Densitometry (BMD)			
Whole body, g/cm^2	0.164 (0.161–0.167)	0.159 (0.158–0.166)	0.9872
Left femur, g/cm^2	0.176 (0.165–0.182)	0.161 (0.158–0.168)	0.0637
Right femur, g/cm^2	0.181 (0.167–0.184)	0.171 (0.159–0.175)*	*0.0394*
Lumbar vertebrae (L3–L5), g/cm^2	0.203 (0.193–0.213)	0.200 (0.195–0.208)	0.8130
Biomechanical testing			
Left femur length, mm	37.22 (36.88–37.67)	36.08 (35.29–36.72)*	*0.0218*
Right femur length, mm	37.31 (36.58–37.96)	36.44 (35.43–37.26)*	*0.0211*
Left femur diameter, mm	3.65 (3.5–3.69)	3.51 (3.37–3.67)	0.1989
Right femur diameter, mm	3.71 (3.58–3.88)	3.65 (3.52–3.82)	0.4056
Maximal load of the left femoral shaft, N	199 (191–217)	201.5 (184.5–220.5)	0.8903
Maximal load of the right femoral shaft, N	183 (179.25–191.25)	196 (188–203.5)	0.1391
Maximal load of left femoral neck, N	150.5 (130–165)	133 (126.5–146.5)*	*0.0494*
Maximal load of right femoral neck, N	152.43 (136–166)	132 (123.7–137.5)*	*0.0486*

Data are expressed as medians (25th–75th percentiles). * $p < 0.05$ (significant values in italics).

a fall, a well-known contributor to fracture risk. The mechanism by which antidepressants impact bone health appears to work primarily via serotonergic pathways [10]. Functional 5-HT transporters and receptors are present in osteoblasts, osteocytes and osteoclasts, and stimulation of these receptors influences bone cell activities [11]. The ergic antidepressants and skeletal effects [12–14]. A previous study has shown that the use of selective serotonin reuptake inhibitors was associated with increased rates of bone loss at the hip in older men [15]. In our study as well, we discovered that mirtazapine, which is a norepinephrine and specific serotonergic antidepressant, has a neg-

and right femurs in the ORX MIRTA group compared with the ORX group.

In the present study, in the ORX MIRTA group compared with the ORX group, there was a significant decrease in the levels of BALP and OPG, both of which are markers of bone formation. In addition, there was a significant increase in levels of sclerostin, which inhibits the activation of osteoblasts. Accordingly, our findings suggest that the increased bone loss with serotonin-norepinephrine antidepressants is mediated via decreased bone formation.

OPG functions as a soluble decoy receptor for the receptor activator of nuclear factor kappa-B ligand (RANKL) and acts by competing with RANKL. RANKL is produced by osteoblastic lineage cells and promotes osteoclast activation, leading to enhanced bone resorption and bone loss. OPG, which is secreted by osteoblastic cells, prevents the interaction of RANKL with its receptor RANK and subsequent stimulation of osteoclast development [7]. BALP is localized in the membranes of osteoblasts, from which it is released into the serum during their activation [17]. Sclerostin is produced by osteocytes and inhibits osteoblast differentiation and bone formation via the Wnt signaling pathway [18]. Downregulation of sclerostin is associated with increased osteogenesis and bone mass [19]. Our findings for OPG, BALP and sclerostin suggest that mirtazapine may suppress bone turnover. We may assume that the reduction in OPG and BALP levels could be explained by the inhibition of osteoblast activity.

There are several limitations that should be considered in evaluating the present study. Firstly, the 3-month follow-up period may be too short for monitoring changes in biomechanical properties of bone tissue. Secondly, the sample size was small; although statistical significance is evident, the capacity to identify possible confounding variables is limited. Finally, behavioral activity was neither controlled nor assessed. In conclusion, the long-term administration of mirtazapine in the orchidectomized rat model can have a negative effect on bone. Reduced BMD, reduced mechanical strength of the bones in the femoral neck, decreased levels of OPG and BALP (markers of bone formation) and increased levels of sclerostin may cause deterioration of the mechanical strength of the bone. Further studies in animals and humans will be needed to confirm these findings.

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Disclosure Statement

The authors declare that they have no conflict of interest.

References

1 Kinjo M, Setoguchi S, Schneeweiss S, Solomon DH: Bone mineral density in subjects using central nervous system-active medications. Am J Med 2005;118:1414.

2 Pack A: Bone health in people with epilepsy: is it impaired and what are the risk factors? Seizure 2008;17:181–186.

3 Hansen DG, Rosholm JU, Gichangi A, Vach W: Increased use of antidepressants at the end of life: population-based study among people aged 65 years and above. Age Ageing 2007;36:449–454.

4 Alam A, Voronovich Z, Carley JA: A review of therapeutic uses of mirtazapine in psychiatric and medical conditions. Prim Care Companion CNS Disord 2013;15:PCC.13r01525.

5 Rabenda VD, Nicolet C, Beaudart O, Bruyère JY: Relationship between use of antidepressants and risk of fractures: a meta-analysis. Osteoporos Int 2013;24:121–137.

6 Borges NC, Barrientos-Astigarraga RE, Sverdloff CE, Donato JL, Moreno P, Felix L, Galvinas PAR, Moreno RA: A fast, sensitive and simple method for mirtazapine quantification in human plasma by HPLC-ESI-MS/MS. Application to a comparative bioavailability study. Biomed Chromatogr 2012;26:1399–1407.

7 Gradosova I, Zivna H, Palicka V, Hubena S, Svejkovska K, Zivny P: Protective effect of amlodipine on rat bone tissue after orchidectomy. Pharmacology 2012;89:37–43.

8 Vanderschueren D, Vandenput L, Boonen S, Van Herck E, Swinnen JV, Bouillon R: An aged rat model of partial androgen deficiency: prevention of both loss of bone and lean body mass by low-dose androgen replacement. Endocrinology 2000;141:1642–1647.

9 Gentile MA, Nantermet PV, Vogel RL, Phillips R, Holder D, Hodor P, Cheng C, Dai H, Freedman LP, Ray WJ: Androgen-mediated improvement of body composition and muscle function involves a novel early transcriptional program including IGF-1, mechano growth factor, and induction of β-catenin. J Mol Endocrinol 2010;44:55–73.

10 Shea MLO, Garfield LD, Teitelbaum S, Civitelli R, Mulsant BH, Reynolds CF 3rd, Dixon D, Doré P, Lenze EJ: Serotonin-norepinephrine reuptake inhibitor therapy in late-life depression is associated with increased marker of bone resorption. Osteoporos Int 2013;24:1741–1749.

11 Moura C, Bernatsky S, Abrahamowicz M, Papaioannou A, Bessette L, Adachi J, Goltzman D, Prior N, Kreiger N, Towheed T, Leslie WD, Kaiser S, Ioannidis G, Pickard L, Fraser LA, Rahme E: Antidepressant use and 10-year incident fracture risk: the population-based Canadian Multicentre Osteoporosis Study (CaMoS). Osteoporos Int 2014;25:1473–1481.

12 Warden JS, Nelson IR, Fuchs RK, Bliziotes MM, Turner CH: Serotonin (5-hydroxytryptamine) transporter inhibition causes bone loss in adult mice independently of estrogen deficiency. Menopause 2008;15:1176–1183.
13 Warden SJ, Robling AG, Haney EM, Turner CH, Bliziotes MM: The emerging role of serotonin (5-hydroxytryptamine) in the skeleton and its mediation of the skeletal effects of low-density lipoprotein receptor-related protein 5 (LRP5). Bone 2010;46:4–12.
14 Bonnet N, Bernard P, Beaupied H, Bizot JC, Trovero F, Courteix D, Benhamou CL: Various effects of antidepressant drugs on bone microarchitecture, mechanical properties and bone remodeling. Toxicol Appl Pharmacol 2007;221:111–118.
15 Haney EM, Chan BK, Diem SJ, Ensrud KE, Cauley JA, Barrett-Connor E, Orwoll E, Bliziotes MM: Association of low bone mineral density with selective serotonin reuptake inhibitor use by older men. Arch Intern Med 2007;167:1246–1251.
16 Horcajada-Molteni MN, Davicco MJ, Coxam V, Lebecque P, Dominguez B, Ritz P, Culioli J, Barlet JP: Treadmill running starting 3 months after orchidectomy restores femoral bone mass in rats. Eur J Appl Physiol Occup Physiol 1999;79:251–259.
17 Moraes RB, Corrêa L, Luz JG: Adverse effects of the amlodipine on bone healing of the mandibular fracture: an experimental study in rats. Oral Maxillofac Surg 2010;15:93–101.
18 Bhattoah HP, Wamwaki J, Kalina E, Foldesi R, Balogh A, Antal-Szalmas P: Serum sclerostin levels in healthy men over 50 years of age. J Bone Miner Metab 2013;31:579–584.
19 Compton JT, Lee FY: A review of osteocyte function and the emerging importance of sclerostin. J Bone Joint Surg Am 2014;96:1659–1668.

11.5. Appendix 5

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Pulmonary, gastrointestinal and urogenital pharmacology

Negative effect of serotonin–norepinephrine reuptake inhibitor therapy on rat bone tissue after orchidectomy

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ABSTRACT

Our goal was to determine if venlafaxine has a negative effect on bone metabolism.

Rats were divided into three groups. The sham-operated control group (SHAM), the control group after orchidectomy (ORX), and the experimental group after orchidectomy received venlafaxine (VEN ORX) in standard laboratory diet (SLD) for 12 weeks. Bone mineral content (BMC) was measured by dual energy X-ray absorptiometry (DXA). Bone marker concentrations of carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), osteoprotegerin (OPG), amino-terminal propeptide of procollagen type I (P1NP), bone alkaline phosphatase (BALP), sclerostin and bone morphogenetic protein 2 (BMP-2) were examined in bone homogenate. The femurs were used for biomechanical testing.

Compared to the ORX group we found lower BMD in the diaphysis area of the femur in the VEN ORX group, suggesting a preferential effect on cortical bone. Of the bone metabolism markers, there was significant decrease (ORX control group versus VEN ORX experimental group) in BALP levels and increase in sclerostin and CTX-I levels, suggesting a decrease in osteoid synthesis and increased bone resorption.

The results suggest that the prolonged use of venlafaxine may have a negative effect on bone metabolism. Further studies are warranted to establish whether venlafaxine may have a clinically significant adverse effect on bone.

1. Introduction

In the last 15 years bone researchers have suggested the importance of the central nervous system in bone metabolism (Takeda et al., 2002; Elefteriou, 2005). Concerns have been raised about the safety of serotonin reuptake inhibitor (SRI) antidepressants in older adults, particularly whether these medications may increase the risk of osteoporosis, falls, and fracture (Kvelde et al., 2013; Rabenda et al., 2013; Cizza, 2011; Darowski et al., 2009). Several categories of drugs that act on the central nervous system such as antidepressants, anticonvulsants and opioids have been shown to increase the risk of fracture and/or osteoporosis (Pack, 2008; Kinjo et al., 2005). Evidence from psychiatric morbidity surveys suggests that the current level of major depression in the general population is about 5% but is higher in the older age groups; antidepressants are thus one of the most commonly prescribed drugs, particularly in people aged 65+ (Hansen et al., 2007). Nearly all previous investigations into the use of these drugs and the risk of fracture or/and bone loss are cross-sectional studies with inadequate control of confounders. Hence there is a strong need for prospective studies to prove a causal link between the medication and adverse effect on bone.

Venlafaxine is a phenethylamine derivative widely prescribed for the treatment of depression, and its mechanism of action is based on the inhibition of the reuptake of serotonin and noradrenaline (SNRI). Venlafaxine's efficacy is comparable with tricyclic anti-depressants; however, the SNRI has fewer adverse effects. As such, the use of venlafaxine has increased in recent years (Ebrahimi et al., 2015). Although its potency at the 5-HTT is less than that of other SSRIs, venlafaxine also inhibits the norepinephrine transporter; however, it is considered serotonin-selective because its potency at the 5-HTT is more than 100 times its potency at the norepinephrine transporter (Shea et al., 2013). The mechanism of action of antidepressants in the regulation of bone

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tissue is not fully understood. Recent studies have found that transporters of serotonin may play a role in bone metabolism and that medications which affect these transporter systems may also affect bone metabolism (Rabenda et al., 2013).

We report the impact of venlafaxine on bone mineral density (BMD), bone metabolism markers and the biomechanical properties of bone. We have used the orchidectomized rat model (the model for androgen-deficiency osteoporosis) in which higher sensitivity can be expected for detection of the possible risk of xenobiotics for bone health.

2. Materials and methods

2.1. Animals

All animals received humane care in accordance with the guidelines set by the institutional Animal Use and Care Committee of Charles University, Prague, Faculty of Medicine in Hradec Kralove, Czech Republic. The protocol of the experiment was approved by the same committee. The experiments used eight-week-old male albino Wistar rats (Biotest s.r.o., Konarovice, Czech Republic). The animals were hosted in groups of 4 in plastic cages. During the experimental period the animals were maintained under controlled conventional conditions (12 h light and 12 h dark, temperature 22 ± 2 °C, air humidity 30–70%). Tap water and standard laboratory diet (SLD, VELAS, a.s., Lysa nad Labem, Czech Republic) or SLD enriched with venlafaxine were given ad libitum. The weights of the rats were monitored once a week.

2.2. Experimental design

Rats weighing 200 ± 10 g at the beginning of the experiment were divided into three groups of eight animals:

1. Sham-operated control fed with SLD (SHAM)
2. Orchidectomised control fed with SLD (ORX)
3. Orchidectomised rat fed with SLD enriched with venlafaxine (12 mg/25 g of the diet; venlafaxine TEVA RETARD, Teva Pharmaceuticals s.r.o, Czech republic) (VEN ORX).

At the beginning of the experiment the rats (ORX and VEN ORX) underwent bilateral orchidectomy under ether anesthesia; SHAM rats underwent only scrotal incision. On the second day after operation VEN ORX began to receive SLD enriched with venlafaxine and SHAM and ORX only SLD, both diets ad libitum. After 12 weeks, the animals were sacrificed by exsanguination from the abdominal aorta under ether anesthesia, and the obtained serum was aliquoted and stored at −80 °C for ensuing biochemical analyses. After killed of the rats, both tibiae and femurs were dissected free of soft tissue, wrapped in gauze moistened with saline and frozen to −80 °C till required for analysis.

2.3. Analysis of serum and bone homogenates

Bone homogenate was prepared from the tibiae. The cartilage was removed from the proximal tibia before dissect of 0.1 g, which included minimal or-no bone marrow. This part was homogenized in 1.5 ml of phosphate buffer (PBS, PENTA Prague, Czech Republic) with a MagNA Lyser instrument (Roche Applied Science, Germany) at 6500 rpm for 20 s, and cooled on the MagNA Lyser Cooling Block. This procedure was repeated three times. The raw tissue homogenate was centrifuged at 10,000g at 4 °C for 10 min, and the resulting supernatant was collected and stored at −80 °C.

Levels of the markers carboxy-terminal cross-linking telopeptide of type I collagen (CTX-I), osteoprotegerin (OPG), amino-terminal propeptide of procollagen type I (P1NP), bone alkaline phosphatase (BALP), sclerostin and bone morphogenetic protein 2 (BMP-2) were determined in this bone homogenate. Bone-marker levels were determined using ELISA kits from Uscn Life Science Inc.

2.4. Dual energy X-ray absorptiometry analysis

The rat bone mineral density (BMD, g/cm^2) was measured after sacrifice by means of dual energy X-ray absorptiometry (DXA) on a Hologic Delphi A device (Hologic, MA, USA) at the Osteocentre of the Faculty Hospital Hradec Kralove, Czech Republic. Prior to measurement, a tissue calibration scan was performed with the Hologic phantom for the small animal. Bone mineral densities of the whole body, in the area of the lumbar vertebrae, and in the area of the femur were evaluated by computer using the appropriate software program for small animals (DXA; QDR-4500A Elite; Hologic, Waltham, MA, USA). All animals were scanned by the same operator.

2.5. Biomechanical testing procedure

Mechanical testing of the rat femoral shaft and femoral neck was done with a special electromechanical custom-made testing machine (Martin Kosek & Pavel Trnecka, Hradec Kralove, Czech Republic) according to the method described in our previous report (Gradosova et al., 2012). For the three-point bending test, the femur was placed on a holding device with the two support points 18 mm apart. A small stabilizing preload to 10 N was applied in the anteroposterior direction to fix the bone between the contacts. A constant deformation rate of 6 mm/min was generated until maximal load failure, and breaking strength (maximum load, N) was recorded. The proximal part of the femur was used for shear test of the femoral neck. The diaphysis of the bone was embedded into a container using a methacrylate resin, and a vertical load was applied to the top of the femoral head. A small stabilizing preload to 10 N was applied and increased at a constant speed of 6 mm/min until failure of the femoral neck. The breaking strength (maximum load, N) was recorded by the measuring unit (Digitalanzeiger 9180, Burster Praezisionsmesstechnik GMBH & Co KG, Gernsbach, Germany). All bones were analyzed by the same operator.

2.6. Statistical analysis

Statistical analysis was performed using the program NCSS 9 (Number Cruncher Statistical System, Kaysville, Utah, USA). Analysis was by two-sample *t*-test or eventually nonparametric Mann–Whitney, and Kolmogorov–Smirnov tests. The results are from measurements made after 12 weeks of the experiment, and are presented as the median and the 25th and 75th percentiles.

3. Results

3.1. Body weight and composition and tissue weights

The performed orchidectomy caused a decrease in weight and lean body mass in ORX in comparison with the SHAM group. DXA revealed that the experimental group showed a decrease in fat mass versus the ORX group. There were no significant differences in lean body mass between the experimental group and the ORX group (Table 1).

3.2. Levels of bone markers

Bone markers from specimens of the proximal tibia were measured to assess the effects of orchidectomy and treatment on bone formation.

Table 1
Body weight and fat mass at the end of the experiment.

Parametr	SHAM	ORX	VEN ORX
Weight (g)	562 (541–622)	474 (460.25–490)[a]	434.5 (428–447)[b]
Fat (%)	19.6 (16.95–20.85)	23 (20.825–28.875)[a]	21.2 (17.5–23.3)
Lean body mass (g)	368.7 (359.85–399.35)	285.4 (278.6–303.3) [a]	294.1 (288.9–309.8)

Data are expressed as medians (25th–75th percentiles).
[a] $P < 0.05$ SHAM versus ORX.
[b] $P < 0.05$ ORX versus VEN ORX.

Table 2
Levels of bone markers.

Parametr	SHAM	ORX	VEN ORX
OPG (pg/ml)	2.23 (2.12–2.46)	1.92 (1.56–2.08)[a]	1.64 (1.53–1.85)
SCLEROSTIN (ng/ml)	0.268 (0.221–0.279)	0.344 (0.288–0.381)[a]	0.457 (0.405–0.484)[b]
P1NP (pg/ml)	217.21 (164.71–248.82)	201.92 (182.58–235.28)	210.21 (175.53–261.09)
BALP (ng/ml)	20.09 (17.21–20.51)	18.31 (17.04–22.30)	7, 15 (5.60–8.87)[b]
BMP-2 (pg/ml)	25.14 (21.78–26.65)	24.88 (22.42–27.57)	22.22 (19.89–23.78)
CTX-I (pg/ml)	56.02 (47.72–61.23)	68.27 (48.72–78.41)	99.54 (79.09–110.30)[b]

Data are expressed as medians (25th–75th percentiles).
BALP—bone alkaline phosphatase; BMP-2—bone morphogenetic protein; OPG—osteoprotegerin; P1NP—aminoterminal propeptide of procollagen type 1.
CTX-I—carboxy-terminal cross-linking telopeptide of type I collagen.
[a] $P < 0.05$.
[b] $P < 0.05$ ORX versus VEN ORX.

3.2.1. *ORX group versus SHAM group*

Determination of the levels of bone turnover markers (OPG) revealed a decrease in ORX versus SHAM. Levels of sclerostin were significantly increased.

3.2.2. *ORX group versus VEN ORX group*

The levels of BALP were decreased, while the levels of CTX-I and sclerostin were increased (Table 2).

3.3. *Dual energy X-ray absorptiometry*

3.3.1. *ORX group versus SHAM group*

In ORX, a significant decrease was demonstrated in whole body BMD and also in the area of the lumbar vertebrae and both femurs compared with SHAM.

3.3.2. *ORX group versus VEN ORX group*

In the VEN ORX group there was a significant decrease in the BMD and BMC of both femurs (Table 3).

3.4. *Biomechanical testing measurement*

3.4.1. *ORX group versus SHAM group*

As we expected, testing of the mechanical strength of the bone tissue by means of three-point bending revealed a statistically significant decrease in maximal load values in ORX versus SHAM. A significant decrease was also observed in the length of both femurs in the ORX group.

3.4.2. *ORX group versus VEN ORX group*

There were no significant differences in biomechanical properties between the experimental group and the control group (Table 4).

4. Discussion

Osteoporosis is a major economic and social burden. Research to identify risk factors of osteoporosis could help in the reduction of its incidence, resulting in a significant public health benefit. To our knowledge, this study is the first preclinical research to examine the effects of venlafaxine on bone metabolism in rats.

At the end of the experiment the level of venlafaxine in the VEN ORX group was 0.46 mg/l (0.3–0.625 mg/l), comparable to therapeutic levels of the drug (0.2–0.75 mg/l). In rats 12 weeks post-orchidectomy we found a significant decrease in body weight, bone mineral density, lean body mass and bone length, and an increase in fat mass. These results confirm the findings from previous studies in rats that deficiency of androgens negatively affects body composition, and establishes these animals as suitable models for investigating androgenic modulation of body composition (Vanderschueren et al., 2000; Gentile et al., 2010; Zhang et al., 1999). However, in the VEN ORX group these changes were not demonstrated compared with the ORX group.

In our study we found that treatment with venlafaxine, a serotonin–norepinephrine reuptake inhibitor, was associated with increased levels of CTX-I, a marker of bone resorption, without a compensatory increase in P1NP, but there were decreased levels of BALP, a marker of bone formation and mineralization. Our findings suggest that the increased rate of bone loss with venlafaxine is mediated via increased bone resorption and also decreased osteoblast differentiation. Among other things, we found increased levels of sclerostin. Thus we confirm the results of previous studies (Shea et al., 2013; Haney et al., 2007; Cauley et al., 2005; Richards et al., 2007; Warden et al., 2010, 2008). Serotonergic antidepressants may increase bone resorption via the serotonin transporter or other types of serotonin receptors (Battaglino et al., 2004). At the same time inhibition of the 5-HTT may have significant damaging effects on bone mineral deposition in the growing mouse skeleton (Warden et al., 2010). BALP is localized in the membranes of osteoblasts, from which it is released into the serum during their activation (Moraes et al., 2010). Sclerostin is produced by osteocytes and inhibits osteoblast differentiation and bone formation via the Wnt signaling pathway (Bhattoa et al., 2013). Upregulation of

Table 3
Comparison of values from DXA analysis.

Parametr	SHAM	ORX	VEN ORX
Whole body BMD (g/cm²)	0.184 (0.181–0.185)	0.164 (0.161–0.167)[a]	0.158 (0.156–0.165)
Whole body BMC (g)	15.893 (15.267–16.282)	12.846 (12.747–13.394)[a]	12.366 (11.859–13.011)
LF BMD (g/cm²)	0.194 (0.191–0.206)	0.176 (0.165–0.182)[a]	0.154 (0.151–0.157)[b]
LF BMC (g)	0.266 (0.253–0.285)	0.228 (0.217–0.235)[a]	0.172 (0.161–0.181)[b]
RF BMD (g/cm²)	0.199 (0.197–0.214)	0.181 (0.167–0.184)[a]	0.161 (0.158–0.166)[b]
RF BMC (g)	0.272 (0.264–0.282)	0.224 (0.216–0.235)[a]	0.176 (0.167–0.183)[b]
Lumbal columna (L3–L5) BMD (g/cm²)	0.248 (0.234–0.251)	0.203 (0.193–0.213)[a]	0.202 (0.198–0.211)
Lumbal columna (L3–L5) BMC (g)	0.768 (0.731–0.790)	0.583 (0.557–0.643)[a]	0.599 (0.561–0.638)

Data are expressed as medians (25th–75th percentiles).
BMD—bone mineral density.
BMC—bone mineral content.
LF—left femur.
RF—right femur.
[a] $P < 0.05$.
[b] $P < 0.05$ ORX versus VEN ORX.

Table 4
Values of biomechanical testing.

Parametr	SHAM	ORX	VEN ORX
LF length (mm)	39 (38.27–39.24)	37.22 (36.88–37.67)[a]	37.24 (36.88–37.83)
RF length (mm)	39.02 (38.44–39.25)	37.31 (36.58–37.96)[a]	37.08 (36.85–37.61)
LF diameter (mm)	3.77 (3.64–3.83)	3.65 (3.5–3.69)	3.51 (3.39–3.54)
RF diameter (mm)	3.79 (3.72–3.9)	3.71 (3.58–3.88)	3.54 (3.50–3.62)
Maximal load of the left femoral shaft (N)	227 (224–236.5)	199 (191–217)[a]	190.5 (177.25–222.5)
Maximal load of the right femoral shaft (N)	217.4 (198–225)	183 (179.25–191.25)[a]	191 (183.75–213.5)
Maximal load of left femoral neck (N)	171 (139.5–184)	150.5 (130–165)[a]	124 (111–133.5)
Maximal load of right femoral neck (N)	160 (143–181.5)	152.43 (136–166)[a]	124.5 (121.75–133.25)

[b] $P < 0.05$ ORX versus VEN ORX.
Data are expressed as medians (25th–75th percentiles).
LF—left femur.
RF—right femur.
[a] $P < 0.05$.

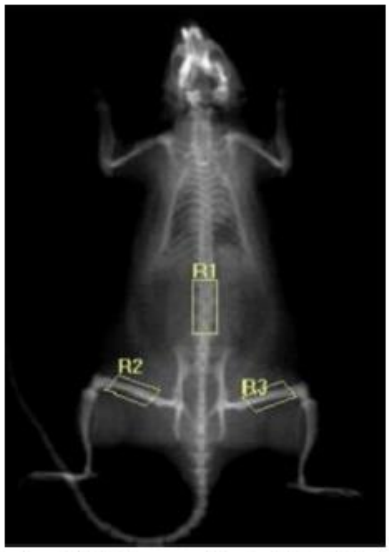

Fig. 1. Evaluation of BMD in three areas of the rat skeleton R1—lumbar columna (L3–L5); R2—left femur; R3—right femur.

sclerostin is associated with decreased osteogenesis and bone mass (Compton and Lee, 2014). These findings explain the decreased levels of BALP at increased levels of sclerostin. CTX-I is released from the C-terminal part of telopeptide collagen I by proteolytic enzymes during its degradation into the systemic circulation, and it is thus a sensitive marker of bone resorption.

Venlafaxine acts via the 5-HTT (5-hydroxytryptamine transporter). The mechanism by which antidepressants impact bone health appears to be primarily via serotonergic pathways (Shea et al., 2013). Functional 5-hydroxytryptamine (5-HT) transporters and receptors are present in osteoblasts, osteocytes and osteoclasts, and stimulation of these receptors influences bone cell activities (Moura et al., 2014). The mechanism of action of different classes of antidepressants appears to produce different skeletal effects: several rodent and human observational studies have reported negative skeletal effects with serotonergic antidepressants. There have been several recent studies linking SSRI/SNRI drugs to increased fragility in humans, which are consistent with our results (Warden et al., 2010, 2008; Bonnet et al., 2007; Lanteigne et al. 2015; Moura et al., 2014).

There are several limitations that should be considered in evaluating the present study. First, the sample size was small: although statistical significance is evident, the capacity to identify possible variables of confusion is limited. Second, the orchidectomy model was used in young, growing rats and it is not known whether the SNRI selected would have similar negative effects on bone in aged rats.

Decreased levels of BALP (markers of bone formation) and increased levels of CTX-I and sclerostin and last but not least, reduced bone density in the diaphysis of both femurs may cause deterioration in the mechanical strength of the bone. This finding is of interest, given the frequent prescription of SSRI to children, adolescents and adults for the treatment of depression and other affective disorders. Notwithstanding, it is also likely that untreated depression has negative effects on bone health. However, treatment with serotonergic antidepressants may require closer monitoring of bone health. Additional longer-term studies are needed to evaluate the interactions between depression, serotonergic antidepressant use, and bone turnover marker and bone density change, and determine their definitive impact on bone health. It can then be assessed who is at high risk from these impacts (Fig. 1).

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References

Battaglino, R., Fu, J., Spate, U., Ersoy, U., Joe, M., Sedaghat, L., Stashenko, P., 2004. Serotonin regulates osteoclast differentiation through its transporter. J. Bone Miner. Res. 19, 1420–1431.

Bhattoa, H.P., Wamwaki, J., Kalina, E., Foldesi, R., Balogh, A., Antal-Szalmas, P., 2013. Serum sclerostin levels in healthy men over 50 years of age. J. Bone Miner. Metab. 31 (5), 579–584.

Bonnet, N., Bernard, P., Beaupied, H., Bizot, J.C., Trovero, F., Courteix, D., Benhamou, C.L., 2007. Various effects of antidepressant drugs on bone microarchitectecture, mechanical properties and bone remodeling. Toxicol. Appl. Pharmacol. 221 (1), 111–118.

Cauley, J.A., Fullman, R.L., Stone, K.L., Zmuda, J.M., Bauer, D.C., Barrett-Connor, E., Ensrud, K., Lau, E.M., Orwoll, E.S., 2005. Factors associated with the lumbar spine and proximal femur bone mineral density in older men. Osteoporos Int. 16, 1525–1537.

Cizza, G., 2011. Major depressive disorder is a risk factor for low bone mass, central obesity, and other medical conditions. Dialogues Clin. Neurosci. 13, 73–87.

Compton, J.T., Lee, Y.F., 2014. A review of osteocyte function and the emerging importance of sclerostin. J. Bone Joint Surg. 96 (19), 1659–1668.

Darowski, A., Chambers, S.A., Chambers, D.J., 2009. Antidepressants and falls in the elderlyDrugs Aging 26381–394

Ebrahimi, M., Ebrahimitalab, A., Es'haghi, Z., Mohammadinejad, A., 2015. Magnetized silane-coupling agent KH-570 based solid-phase extraction followed by gas chromatography–flame ionization detection to determine venlafaxine in human hair and aqueous environmental samples. Arch. Environ. Contam. Toxicol. 68 (2), 412–420.

Elefteriou, F., 2005. Neuronal signaling and the regulation of bone remodeling. Cell. Mol. Life Sci. 62, 2339–2349.

Gentile, M.A., Nantermet, P.V., Vogel, R.L., Phillips, R., Holder, D., Hodor, P., Cheng, C., Dai, H., Freedman, L.P., Ray, W.J., 2010. Androgen-mediated improvement of body composition and muscle function involves a novel early transcriptional program including IGF-1, mechano growth factor, and induction of -catenin. J. Mol. Endocrinol. 44, 55–73.

Gradosova, I., Zivna, H., Palicka, V., Hubena, S., Svejkovska, K., Zivny, P., 2012. Protective effect of amlodipine on rat bone tissue after orchidectomy. Pharmacology 89, 37–43.

Haney, E.M., Chan, B.K., Diem, S.J., Ensrud, K.E., Cauley, J.A., Barrett-Connor, E., Orwoll, E., Bliziotes, M.M., 2007. Association of low bone mineral density with selective serotonin reuptake inhibitor use by older men. Arch. Intern. Med. 167, 1246–1251.

Hansen, D.G., Rosholm, J.U., Gichangi, A., Vach, W., 2007. Increased use of antidepressants at the end of life: population-based study among people aged 65 years and above. Age Ageing 36 (4), 449–454.

Kinjo, M., Setoguchi, S., Schneeweiss, S., Solomon, D.H., 2005. Bone mineral density in subjects using central nervous system-active medications. Am. J. Med. 118 (12), 1414.

Kvelde, T., McVeigh, C., Toson, B., 2013. Depressive symptomatology as a risk factor for falls in older people: systematic review and meta-analysis. J. Am. Geriatr. Soc. 61, 694–706.

Lanteigne, A., Sheu, Y.H., Stürmer, T., Pate, V., Azrael, D., Swanson, S.A., Miller, M., 2015. Serotonin–norepinephrine reuptake inhibitor and selective serotonin reuptake inhibitor use and risk of fractures: a new-user cohort study among US adults aged 50 years and older. CNS Drugs, 24, Epub ahead of print.

Moraes, R.B., Corrêa, L., Luz, J.G., 2010. Adverse effects of the amlodipine on bone healing of the mandibular fracture: an experimental study in rats. Oral Maxillofac. Surg. 15, 93–101.

Moura, C., Bernatsky, S., Abrahamowicz, M., Papaioannou, A., Bessette, L., Adachi, J., Goltzman, D., Prior, N., Kreiger, N., Towheed, T., Leslie, W.D., Kaiser, S., Ioannidis, G., Pickard, L., Fraser, L.A., Rahme, E., 2014. Antidepressant use and 10-year incident fracture risk: the population-based Canadian Multicentre Osteoporosis Study (CaMoS). Osteoporos. Int. 25, 1473–1481.

Pack, A., 2008. Bone health in people with epilepsy: is it impaired and what are the risk factors? Seizure 17 (2), 181–186.

Rabenda, V., Nicolet, D., Beaudart, C., 2013. Relationship between use of antidepressants and risk of fractures: a meta-analysis. Osteoporos. Int. 24, 121–137.

Richards, J.B., Papaioannou, A., Adachi, J.D., Joseph, L., Whitson, H.E., Prior, J.C., Goltzman, D., 2007. Effect of selective serotonin reuptake inhibitors on the risk of fracture. Arch. Intern. Med. 167, 188–194.

Shea, M.L., Garfield, L.D., Teitelbaum, S., Civitelli, R., Mulsant, B.H., Dixon, D., Doré, P., Lenze, E.J., 2013. Serotonin–norepinephrine reuptake inhibitor therapy in late-life depression is associated with increased marker of bone resorption. Osteoporos. Int. 24 (5), 1741–1749.

Takeda, S., Elefteriou, F., Levasseur, R., Liu, X., Zhao, L., Parker, K.L., Armstrong, D., Ducy, P., Karsenty, G., 2002. Leptin regulates bone formation via the sympathetic nervous system. Cell 111, 305–317.

Vanderschueren, D., Vandenput, L., Boonen, S., Van Herck, E., Swinnen, J.V., Bouillon, R., 2000. An aged rat model of partial androgen deficiency: prevention of both loss of bone and lean body mass by low-dose androgen replacement. Endocrinology 141, 1642–1647.

Warden, J.S., Nelson, I.R., Fuchs, R.K., Bliziotes, M.M., Turner, C.H., 2008. Serotonin (5-hydroxytryptamine) transporter inhibition causes bone loss in adult mice independently of estrogen deficiency. Menopause 15 (6), 1176–1183.

Warden, J.S., Roblingb, A.G., Haneyc, E.M., Turnerd, CH.H., Bliziotesc, M.M., 2010. The emerging role of serotonin (5-hydroxytryptamine) in the skeleton and its mediation of the skeletal effects of low-density lipoprotein receptor-related protein 5 (LRP5). Bone 46 (1), 4–12.

Zhang, X.Z., Kalu, D.N., Erbas, B., Hopper, J.L., Seeman, E., 1999. The effects of gonadectomy on bone size, mass, and volumetric density in growing rats are gender-, site-, and growth hormone-specific. J. Bone Miner. Res. 14 (5), 802.