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

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1. SOUHRN

Zvýšení průměrné délky života světové populace je spojeno se zdravotními problémy. Jedním z těchto problémů, který se často vyskytuje obzvláště u starších lidí je snížení kvantity a kvality kostní hmoty nebo-li osteoporóza. Je to systémové skeletální onemocnění způsobené úbytkem kostní hmoty a u žen i mužů představuje významný zdravotní problém. Je charakterizována nízkou kostní hmotou, poškozením mikroarchitrektury kostní tkáně a zvýšením křehkosti kostí s náchylností ke zlomeninám. Osteoporotické fraktury významně zvyšují morbiditu a mortalitu pacientů. Mezi rizikové faktory, které mohou mít vliv na vznik osteoporózy patří dlouhodobé užívání léků, jako jsou např. antiepileptika a antidepresiva.

Cílem této práce bylo zhodnotit účinek orchidektomie a vliv novějších antiepileptik (levetiracetam, lacosamid, topiramat, lamotriginu) a antidepresiv (mirtazapin, venlafaxinu a trazodonem) na kostní metabolismus u zdravých samců potkanů kmene wistar.

Prvním cílem bylo zjistit vliv orchidektomie na metabolismus kostí u potkanů. Zjistili jsme, že orchidektomie měla negativní vliv na kostní metabolismus u potkanů. Tyto výsledky potvrzují, že tato zvířata jako vhodné modely pro zkoumání androgenní modulace složení těla.

Druhým cílem bylo zjistit vliv vybraných antiepileptik (levetiracetamu, lacosamid, topiramátu, lamotrigin) na kostní metabolismus u potkanů a míru (negativního) vlivu vybraných antiepileptik ve srovnání s kontrolní skupinou. Zjistili jsme, že dlouhodobé podávání levetiracetamu, lacosamidu a topiramatu může mít negativní vliv soudě podle snížení BMD v oblasti femuru. Nicméně, po 12 týdnech výsledky neukázaly žádné snížení biomechanické pevnosti kostí. Na druhé straně, stejně jako snížení BMD, dlouhodobé podávání lamotriginu má za následek taky zhoršení mechanické pevnosti kosti. Prokázali jsme negativní účinek u všech z vybraných antiepileptik. Negativní účinek byl největší pro lamotrigin, a snižoval se postupně v pořadí: lacosamid, topiramat a levetiracetam.

Posledním cílem bylo stanovit vliv vybraných antidepresiv (mirtazapin, venlafaxin, trazodonem) na kostní metabolismus u potkanů a dále nás zajímal rozsah (negativní) vlivu vybraných antidepresiv ve srovnání s kontrolní skupinou. Naše výsledky po 12 týdnech naznačují, že podávání mirtazapinu může potlačit kostní obrat, a to zejména v oblasti krčku femuru. Dlouhodobé podávání venlafaxinu a trazodonu naznačuje inhibici activity osteoblastů. U všech vybraných antidepresiv jsme zjistili prokazatelně negativní vliv na metabolismus kostí u potkanů. Nicméně se objevily rozdíly mezi jednotlivými léky v rozsahu negativního efektu. Aktivita osteoblastů byla narušena nejvíce u trazodonu a nejméně u mirtazapinu. U mirtazapinu se také potvrdila nejvyšším snížením mechanické odolnosti v krčku stehenní kosti.

2. SUMMARY

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. Osteoporosis is a systemic skeletal disorder and is a result of loss of skeletal mass. Osteoporosis is characterized by low bone mass, microarchitectural deterioration of bone tissue and an increase in bone fragility and susceptibility to fracture. Osteoporotic fractures are a significant cause of morbidity and mortality. The longterm use of drugs such as antiepileptics and antidepressants could affect the onset of osteoporosis.

The aim of the study was to evaluate the effect of orchidectomy, the effect of newer antiepileptic (levetiracetam, lacosamide, topiramat, lamotrigine) and antidepressive drugs (mirtazapine, venlafaxine and trazodone) on bone metabolism in healthy male Wistar rats.

The first specific aim 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.

The second specific aim was to determine the effect of selected antiepileptic drugs (levetiracetam, lacosamide, topiramate, lamotrigine) on bone metabolism in rats and the extent of the (negative) effect of selected antiepileptic drugs in comparison to the control group. 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. 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.

The last specific aim was to determine the effect of selected antidepressant drugs (mirtazapine, venlafaxine, trazodone) on bone metabolism in rats and we were interested in the extent of the (negative) effect of selected antidepressant drugs in comparison to the control group. 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. 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.

3. BACKGROUNDS

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.

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)*.

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).*

4. OBJECTIVES

In our study we set out the following 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.

5. MATHERIALS AND METHODS

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°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.

5.1. Experiments

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)

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)

5.2. Analysis

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).

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, Venlafaxine and Trazodone**

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).*

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.

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.

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 $p < 0,05$

6. RESULTS

6.1. 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.

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.

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.

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.

6.2. 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.

Levels of bone markers

Levetiracetam administration for 12 weeks caused a significant decrease in OPG and a borderline-significant increase in CTX-I. P1NP 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.

Biomechanical Properties

There was no statistically significant difference in these parameters between rats receiving levetiracetam and control rats.

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.

Levels of bone markers

Among the tested bone markers aminoterminal propeptide of procollagen type 1 was significantly lower in the LCM-ORX group.

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.

Biomechanical Properties

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

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.

Levels of bone markers

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.

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.

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.

Levels of bone markers

No statistically significant difference in bone turnover markers was found.

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.

Biomechanical Properties

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

6.3. 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.

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.

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.

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.

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.

Levels of bone markers

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

Dual Energy X-Ray Absorptiometry

In the group VENLA-ORX there was a significant decrease in the BMD and BMC of both femurs.

Biomechanical Properties

There were no significant differences in biomechanical testing measurement between the experimental group and the control group.

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.

Levels of bone markers

The levels of OPG and BALP were decreased. The levels of sclerostin were increased.

Dual Energy X-Ray Absorptiometry

There were no significant differences in BMD and BMC between the experimental group and the control group.

Biomechanical Properties

There were no significant differences in biomechanical testing measurement between the experimental group and the control group.

7. 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).*

7.1. 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).*

7.2. 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.

7.3. The effect of the selected newer antidepressant drugs on 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.

8. 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.

9. LITERATURE

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PUBLICATIONS AND LECTURES

Original papers

- **Fekete S**, Simko J, Mzik M, Karesova I, Zivna H, Zivny P, Pavliková L, Palicka V. Negative effect of serotonin-norepinephrine reuptake inhibitor therapy on rat bone tissue after orchidectomy. Eur J Pharmacol. **2015; 15 (761): 65-9**. **IF 2,532.**
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