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The Effect of Raloxifene given in Post Menopausal Osteoporosis against the effects of Anti-Epileptics with the help of DEXA analysis and other Pathways

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ABSTRACT: The disease Epilepsy is known to be a common neurological disorder which is affecting majority of population in the whole world. The disease is caused due to many factors which includes abnormalities in brain, stroke, head injury, drug abuse, brain infection. Therefore, to treat such kind of disorders anti-epileptic drugs are used in major as well as minor quantity. By take excessive intake of anti-epileptic drugs it has shown to reduce bone density and bone mineral concentration in people who are taking these drugs on regular basis. As Anti-Epileptic Drugs have been known to be in association with negative impact on the bone health which leads to many kinds of bone disorders. Many kinds of biochemical abnormalities of bone metabolism are seen in patients taking these drugs. There were many kinds of challenges faced during the study as the Bone Mineral Density done using DEXA analysis was the measurement of soft tissue that is changes in femur and lumbar vertebrae. There was a contribution seen by the university in terms of apparatus/chemicals and other necessary requirements.
As if by seeing all the negative values it is found that the drug named Raloxifene has found to be a pre-eminent gem which has reduced the chances of bone density and other bone related disorders.

Keywords: Raloxifene, Anti-epileptic, DEXA, Sodium Valproate, Phenytoin.

INTRODUCTION

The disease termed as "Epilepsy" is known to be a most common neurologic disorder affecting majority of people in the whole world (Johannessen Landmark and Patsalos 2010). For treating such kind of disease there is need of first line treatment which is done by the administration of anti-epileptic drugs (AEDs) with that they are divided into three generations-first, second third generations. The most frequently used AEDs are Phenytoin (PHT), Pheno-barbital (PB), carbamazepine (CBZ), Valporic acid (VPA) & Clobazam (CLB). According to the International League Against Epilepsy it is known as the disease of the brain which is occurring due to majority of reasons such as at least two unprovoked seizures which are coming more that twenty-four hours apart; in which one is unprovoked seizure with the chances of more seizures over the next years.

Bone Structure and its Metabolism. Bone, a major contributing tissue of body that is perpetually remoulded throughout the life time of the individual (Holick and Krane 2001). There are specialized cells known as osteoblasts they help in initiating the formation of bone in which osteocytes detects bone mechanical stress & osteoclasts helps in bone resorption. The determination of bone density is done by the absolute balance in between the bone formation & bone resorption. The process of bone formation starts with the accumulation of organic matrix through osteoblasts which is accompanied by the process of mineralization. The organic matrix is composed of mainly type I collagen and other proteins.

There are a majority of biochemical markers that can be measured and they show the complete rate of bone remodelling.

Epilepsy: Bone Health in Women. Women are on major urge of epilepsy (Ohta *et al.*, 1992). It is found that the women with epilepsy are more prone and come with more problems in relation to bone health which results with low Bone Mineral Density as compared to males. The deficiency of estrogen which is either due to late in first menstrual cycle, primary / secondary amenorrhea as these are the major risk factors which leads to enhanced bone loss in women.

Many studies show that women lose around 35-50% of their complete bone mass in full period of their life (Riggs *et al.,* 1981). As before menopause this loss is very less and involves particular cancellous bone but during the 3-4 years of preceding menopause as well as

after menopause there is a high rate of bone loss with its degeneration.

Anti-Epileptic Drugs: Association with Bone Disease. In the study of Anti-Epileptic Drugs Many Biochemical, Pathological techniques used to enquire the effects of anti-epileptics on the bone mineral density. Pack and Morrell *et al*. (2004) which is done on the drugs such as Phenytoin, Phenobarbitone, Carbamazepine and many others. These Anti-Epileptics are associated with reduced Bone Mineral Density that are the inducers of hepatic cytochrome P450 system.

There are many Anti-Epileptic Drugs that are linked with modified bone metabolism and reduced bone density that are inducers of cytochrome P450 enzyme system which also includes Phenytoin, Carbamazepine, Phenobarbital and many others (Sheth *et al.,* 1955).

Therefore, by seeing these consequences of AEDs they are found to be causing more number of side effects in the regions of bone and corresponding parts. To clarify such conditions many techniques have been created for detecting histologic & radiographic evidences of bone deformation in people who are taking AEDs which ranges from bone biopsies to the present gold standard in detecting reduced BMD & dual energy X-ray absorptiometry.

The DXA accesses mainly trabecular bone for example the spine and ribs and in that it measures the total mineral concentration (Le Blanc *et al.,* 1986). It is known to be the most sensitive technique which is used for assessing BMD. Many studies have measured BMD in the patients who are taking AEDs by using DXA which has shown reduced values of ribs & spine, femoral neck etc. Many biochemical abnormalities of bone metabolism are seen in people who are taking AEDs. These abnormalities include hypocalcaemia, reduced serum level of active vitamin D metabolites.

It is found that in many people estrogen and progesterone helps in keeping bones strong. It is further found that the production of cytokines (interleukins, IL-6, IL-7, and TNF-), as well as the effects of parathyroid hormone on calcium, is stopped by estrogen. They also help osteocytes become more involved through the regulatory oversight of platelet-derived growth factor, Transforming Growth Factor 3, a bone matrix protease with anti-osteoclastic properties. That is important because it balances the growth of bone matrix by osteoblasts and the removal of bone matrix by osteoclasts thus further the density of bones become stable.

Evaluation & Measurement: Bone Mass. The estimation of bone mass provides a quantitative measurement of present skeletal status and also a baseline for future analysis (Blake and Fogelman 1997). It also helps in confirmation of peoples with osteoporosis. In the measurement of DXA the bone mineral content & the bone area is scanned and expressed in g/square cm which shows the "areal" bone density. The measurement of BMD in the area of lumbar spine & femoral neck are known to be the central sites for the study.

The Research aim is to find out the various consequences of Anti-Epileptic drugs in patients who are suffering from Epilepsy and to treat those conditions of bone with the help of drug Raloxifene which helps to reduce the chances of osteoporosis in post-menopausal women by preventing the thickening of bone and other related parts of bone.

The Objective of the paper is to see how the drug Raloxifene affect the bone density and metabolism which is caused due to AEDs.

MATERIAL AND METHODS

To conduct the research study there was use of various kinds of chemicals such as Phenytoin sodium (Unichem Pvt. Ltd. India), Sodium Valproate (Unichem Pvt. Ltd. India), Levetiracetam (Unichem Pvt. Ltd India), Raloxifene (Dr. Reddy Laboratories Pvt. Ltd India) Calcium and vitamin D3 (Cipla Ltd. India), Vitamin D3 (Mankind Pharma Ltd. India) was purchased different Pharmaceutical Industries. The different chemical and Regents used in the present study were of analytical grade and purchased from a local vendor.

The Experimental Animals used in the study are considered as Pathogens free female Swiss albino mice (25-35g) in the animal's house of Pranveer Singh Institute of Technology, Kalpi Road, Bhaunit, Kanpur-208020 having Registration No-1273/PO/S/09/CPCSEA with the prior approval from Institutional Animal Ethical Committee (IAEC), bearing approval according to CPCSEA guidelines for carrying animal activity. Healthy female Swiss albino mice were used for activity. The animals were housed under standard condition as prescribed and had a proper approach to water and feed with the exclusion of food during the period.

The various methods used in the study involves:

A. Modelling bone loss due to AEDs (PHT, SVP, and LTM)

As the diagnosis of osteoporosis is presently based by measuring the bone mineral density there are other known factors which increase the risk of fractures which is free from Bone Mineral Density (Raisz, 2005) All rats were divided randomly into six group of 10 animals in each group. For 4 months the following Antiepileptic medication were given once day via orally route. PHT (35 mg/kg), SVPL (100 mg/kg), SVPH (300 mg/kg), LTML (100 mg/kg), and LTMH (200 mg/kg). After 3 months of therapy and at plasma levels within the therapeutic range, we found that a previously standardized dose of PHT (35 mg/kg) induced bone loss in the femur of mice. We decided to give the treatment an extra month to see if the lumbar bones had changed. Four months after starting therapy, blood was drawn under ether anaesthesia to separate the serum in order to determine the medication concentration, and urine was taken to estimate urinary calcium. At last, the animals were slaughtered so that bone samples could be taken for histological and biochemical analysis from the femur and lumbar regions.

Group I: - Vehicle (1mg/kg)
Group II: - PHT (35 mg/kg)
Group III: - SVPL (100 mg/kg)
Group IV: - SVPH (300 mg/kg)
Group V: - LTML (100 mg/kg)
Group VI: **-** LTMH (200 mg/kg)

B. Preventive therapy

Twelve groups of ten animals each comprised the experimental population of 120 animals. Oral administration of RLX, CVD, or CVDD preceded administration of AEDs (PHT, SVP, and LTM) now when plasma concentration of the bone protecting medicines reaches C_{max}. PHT and SVP chose AED doses known to cause bone loss.

Group I: -Vehicle (1 ml/kg)
Group II: -RLX (15 mg/kg)
Group III: -PHT (35 mg/kg)
Group IV: -SVP (300 mg/kg)
Group V: -LTM (200 mg/kg)
Group VI: -RLX + PHT (15+35 mg/kg)
Group VII: -RLX + SVP (15+ 300 mg/kg)
Group VIII: -RLX + LTM (15+ 200 mg/kg)
Group IX: -CVD + PHT (130 + 65 mg/kg)
Group X: - CVD + SVP (130 mg/kg + 65 IV) + 300 mg/kg
Group XI: -CVD +LTM (130 mg/kg+ 65 ID) + 200
Group XII: -13 mg/kg + 65 IV)

C. Care for therapeutic purposes

The 120 animals used in the study were split up into 12 groups of 10. Except for the groups, the animals were given AEDs (PHT, SVP, and LTM) for a total of 4 months by oral administration (1, 2 and 12). For an additional month, animals in groups 3, 4, and 11 were given RLX or CVDD, while those in groups 1, 2, and 12 were given simply vehicle. Urine was collected over the course of 24 hours after the conclusion of both the preventative and therapeutic treatments to determine the total amount of calcium in the urine (U-Ca). Serum estradiol levels were determined after an overnight fasting period from the mice (E2). The mice were euthanized as soon as blood was drawn, and their femurs and lumbar spines (L2-L4) were removed for histopathology, biochemical estimations such as alkaline phosphatase (ALP), tartrate resistant acid phosphatase (TRAP), and hydroxyproline (HxP), and bone mineral density (BMD) analysis using a DEXA scan.

Group I: - Vehicle (1mg/kg)
Group II: - RLX (15mg/kg)
Group III: - PHT (35mg/k
Group IV: - SVP (300mg/kg)
Group V: -LTM (200mg/kg)
Group VI: -PHT+RLX (135+15 mg/kg)
Group VII: -SVP + RLX (300+15 mg/kg)
Group VIII: - LTM + RLX (200 +15 mg/kg)
Group IX: -PHT +CVDD 35+ (130mg/kg +65IU) (195IU)
Group X: -SVP + CVDD 300+ (130mg/kg +65 IU) + (195IU)
Group XI: - LTM + CVDD 200 + (130 mg/kg + 65 IU) + (195 IU)
Group XII: -CVDD (130 mg/kg +65 IU) + (195 IU).

D. Electroconvulsive group

Preceding psychopharmacological treatment by approximately 15 years, electroconvulsive therapy (ECT) is the first scientifically developed method for treatment of psychological disorders and remains an important method today in the face of all prejudices (Aki *et al.*, 2013). In order to determine the impact of Raloxifene on the efficiency of AEDs in a model of seizure control, mice were separated into twelve groups of ten animals each and challenged with electroshock following chronic therapy.

Group I: - Vehicle (1mg/kg)
Group II: - RLX (15 mg/kg)
Group III: - PHT (35 mg/kg)
Group IV: - SVP (300mg/kg)
Group V: - LTM (200mg/kg)
Group VI: - PHT+RLX (135+15 mg/kg)
Group VII: - SVP + RLX (300+15 mg/kg)
Group VIII: -LTM +RLX (200+15 mg/kg)
Group IX: - PHT + CVD 35+ (130 mg/kg+ 65 IV)
Group X: - SVP + CVD 300 + (130 mg/kg+65 IU)
Group XI: - LTM +CVD 200+ (130 mg/kg +65 IU)
Group XII: -SVP + RLX (300+15 mg/kg)

Further conduction of study was done on Experimental Model which includes:

Electroshock induced seizures. Using an electro-convulsometer, mice were subjected to induced convulsions. The ear-clip electrodes were used to deliver an alternating current (with a maximum stimulation voltage of 500 V and a fixed current intensity of 21 mA for 0.2 seconds). The maximum range of motion of the hind limbs was determined to be tonic extension, which is defined as an extension of 180 degrees from the plane of the body axis.

Bone Mineral Density and Bone Mineral Content analysis by DEXA. Soft tissue was removed from the left femur and lumbar vertebrae (L2-L4) before they were frozen at -80 degrees Celsius. After defrosting for 30 minutes, the bones were scanned using DEXA for BMD analysis.

The Sample prepared for the study includes;

Bone tissue sample preparation. Muscles and other tissues were cut away from the lumbar vertebrae (L2-L4) and the bones were extracted. The bones were weighed separately and then homogenized in a triethanolamine buffer of 10 mM volume (pH 7.5). After homogenizing for 1.5 hours at 4 C with stirring, the mixture was centrifuged. Bone extracts were processed twice to ensure enough quantities for measuring alkaline phosphatase (ALP) and tartrate resistant acid phosphatase (TRAP) activity. To determine the amount of hydroxyproline (HxP), the insoluble pellets were heated to 105 degrees Celsius in 6N HCl for 24 hours.

Serum preparation. Each animal's blood sample (about 2.5 times the volume needed for usage) was incubated at room temperature, upright, for 45 minutes. The samples were centrifuged at 3,000 rpm for 10 minutes after the clot had retracted. The resulting serum was frozen in liquid nitrogen for later use.

Urine Collection. The urine was collected for 24 hours and then frozen at -20 degrees Celsius in a sterile flask.

RESULTS AND DISCUSSION

The findings done in the study are seen through Dexa investigations as;

A. BMD: Bone mineral Density

As known that Vitamin D supplements are known to be given for all with using Anti-Epileptic Drugs mainly for those who have less level of vitamin D & are at more risk of diseases related to bone Mikati *et al.* (2006).

Bone mineral density was measured using DEXA and was shown to have changed in the femur and lumbar vertebrae (L2-L4), (BMD). Bone mineral density in the lumbar vertebrae and femur mice treated with 35 mg/kg

of PHT and 300 mg/kg of SVP for four months was significantly lower than that of control animals. However, SVP (100 mg/kg) did not affect femoral bone mineral density (BMD), primarily on lumbar spine BMD. There was no difference in bone mineral density between the 100 mg/kg and 200 mg/kg LTM groups after four months of therapy (BMD).

B. BMC: Bone mineral Concentration

Chronic therapy with PHT (35 mg/kg) decreased BMC significantly over four months. However, SVP (100, 300 mg/kg) and LTM (100, 200 mg/kg) did not affect BMC.

C. Concentrations of antiepileptic drugs in plasma

Blood drug concentrations were found to be within the therapeutic range (10-20 g/ml) after four months of PHT (35 mg/kg). As a bonus, serum medication concentrations at 100 and 300 mg/kg SVP were within the human therapeutic range (50-100 mg/ml). With 200 mg/kg, serum concentrations of LTM were within the therapeutic range (12-46 g/ml), but not with 100 mg/kg.

D. Reversing and Preventing Bone Loss from PHT and SVP with Raloxifene

BMD: Density of bone tissue

Four months after treatment, PHT (35 mg/kg) and SVP (100 and 300 mg/kg) significantly decreased bone mineral density (BMD) in the lumbar vertebrae and femurs of PHT- and SVP-treated mice compared to controls. When RLX was stopped, bone mineral density in the femur and lumbar spine returned to its pre-PHT and post-SVP values. Although CVD prevented further loss of BMD, it was not as effective as RLX (p=0.01) or RLX + RLX (p=0.001) in restoring the lumbar vertebrae after PHT and SVP treatment or in the femur after SVP treatment, in restoring lost BMD.

BMC: Bone Mineral Content. Mice given PHT (35 mg/kg) and SVP (100 and 300 mg/kg) for four months had significantly lower bone mineral density (BMD) in the lumbar vertebrae and femurs compared to the control group. When RLX was inhibited, bone mineral density (BMD) in case of femur & lumbar spine in comparison with the control group. Compared to RLX, CVD preventive treatment was significantly less effective in restoring bone mineral density in the lumbar vertebrae after PHT and SVP and in the femur after SVP treatment. One month after injection, RLX and CVDD (treatment) yielded results equivalent to PHT and SVP.

Table 1: Plasma levels of antiepileptic medicines (PHT, SVP, and LTM) after 16 weeks of treatment.

Groups	Control	PHT	SVP		LTM	
Weight (g)	29.2±0.59	27.4 ± 1.08	Low dose	High dose	Low dose	High dose
			27 ± 0.59	26.3 ± 0.96	28.9 ±0.76	27.9 ± 0.67
Dose (mg/kg)	-	35	100	300	100	200
Plasma drug Conc. (µg/ml)	-	18.46 ±0.71	51.26 ± 1.1	98.92 ± 1.59	10.85 ± 1.14	42.28 1.23

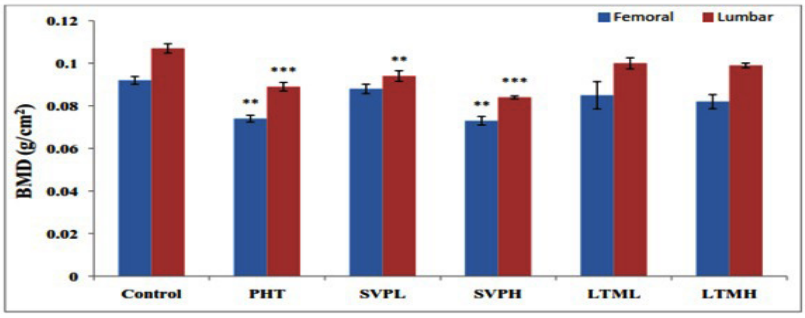

Fig. 1. Bone mineral density (BMD) in mice's femur and lumbar vertebrae was analysed after exposure to phenytoin, sodium valproate, and levetiracetam.

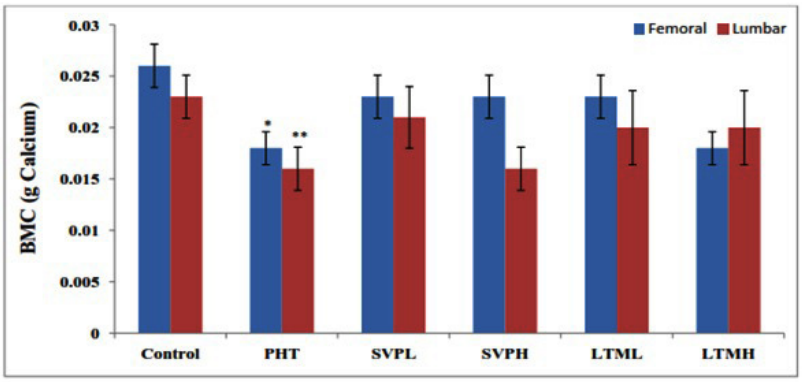

Fig. 2. Bone mineral content (BMC) of mouse femurs and lumbar vertebrae after treatment with phenytoin, sodium valproate, and levetiracetam.

Over four months, we looked at how raloxifene affected people with seizures caused by electroshock, both on its own and in combination with either PHT or SVP. We found that raloxifene (RLX) caused seizures when electroshock (ES) was used, but it did not change HLE significantly. It was found that RLX ability to survive from status epilepticus in female mice in full condition. This shows that long-term use of RLX does not change how well PHT, SVP, or LTM work to stop seizures.

E. Bone changes caused by AEDs and the influence of raloxifene/CVD/CVDD

After four months of treatment with PHT and SVP, bone changes were seen in female Swiss albino mice. This means that mice can be used to test possible drugs for treating bone loss. More clinical research is needed to confirm this. Still, LTM may be a safer option for women with epilepsy prone to osteoporosis or with risk factors because it does not change the histology, bone density (BMD), or bone metabolism markers.

CONCLUSIONS

The above results show that Raloxifene helps to prevent and treat the bone loss which is caused due to Phenytoin & Sodium Valproate as they showed a great changes of side effects on bone health and as well showed how this drug helped to prevent or stop seizures. It is shown that how raloxifene has the ability to survive from status epilepticus in female mice.

FUTURE SCOPE

The effective activity of Raloxifene is found to be of major use in case of Epileptic women's who are under administration of anti-epileptic drugs as it helped to reduce the chances of post-menopausal osteoporosis by effectively acting on the bone strength and bone modelling.

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Conflict of Interest. None.

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