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Raloxifene preserves phenytoin and sodium valproate induced bone loss by modulating serum estradiol and TGF-β3 content in bone of female mice

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ABSTRACT

Antiepileptic drugs (AEDs)-induced adverse consequences on bone are now well recognized. Despite this, there is limited data on the effect of anti-osteoporotic therapies on AEDs-induced bone loss. We hypothesize that estrogen deprivation following phenytoin (PHT) and sodium valproate (SVP) therapy could lead to adverse bony effects. Both PHT and SVP inhibit human aromatase enzyme and stimulate microsomal catabolism of oestrogens. Estrogen deficiency states are known to reduce the deposition of transforming growth factor-β (TGF-β3), a bone matrix protein, having anti-osteoclastic property. Thus, an attempt was made to investigate the effect of raloxifene, a selective oestrogen receptor modulator, in comparison with calcium and vitamin D3 (CVD) supplementation, on PHT and SVP-induced alterations in bone in mice and to unravel the role of estradiol and TGF-β3 in mediation of bony effects by either AEDs or raloxifene. Further, the effect of raloxifene on seizures and on the antiepileptic efficacy of PHT and SVP was investigated. Swiss strains of female mice were treated with PHT (35 mg/kg, p.o.) and SVP (300 mg/kg, p.o.) for 120 days to induce bone loss as evidenced by reduced bone mineral density (BMD) and altered bone turnover markers (BTMs) in lumbar bones (alkaline phosphatase, tartarate resistant acid phosphatase, hydroxyproline) and urine (calcium). The bone loss was accompanied by reduced serum estradiol levels and bone TGF-β3 content. Preventive and therapeutic treatment with raloxifene ameliorated bony alterations and was more effective than CVD. It also significantly restored estradiol and TGF-β3 levels. Deprived estrogen levels (that in turn reduced lumbar TGF-β3 content) following PHT and SVP, thus, might represent one of the various mechanisms of AEDs-induced bone loss. Raloxifene preserved the bony changes without interfering with antiepileptic efficacy of these drugs, and hence raloxifene could be a potential therapeutic option in the management of PHT and SVP-induced bone disease if clinically approved.

1. Introduction

Epileptic patients are at an increased risk of metabolic bone disease and fractures; not only epilepsy but also antiepileptic drugs (AEDs) intricately modulate the bone microarchitecture and bone mineral density (BMD) and increase the incidence of fractures (Lee et al., 2010; Valsamis et al., 2006). Multiple factors may contribute and hence the gross bony changes and increased risk of fractures are not solely attributable to AEDs but also to seizure activity associated falls, trauma and sedentary lifestyle (Khanna et al., 2009). While the mechanisms responsible for AED-related bone fragility are probably multiple and still inadequately understood, AEDs most commonly reported to cause disorders of bone metabolism are potent inducers of the cytochrome P450 (CYP 450) monoxygenase system (phenytoin; carbamazepine; phenobarbitone) that influences calcium–vitamin D axis by reducing bio-available vitamin D resulting in hypocalcemia and compensatory secondary hyperparathyroidism that restores calcium levels both rapidly by increasing the reabsorption of calcium

Abbreviations: AED, antiepileptic drug; ALP, alkaline phosphatase; BMD, bone mineral density; BTMs, bone turnover markers; CVD, calcium vitamin D; CYP450, cytochrome P450; DEXA, dual energy X- ray absorptiometry; HxP, hydroxyproline; PHT, phenytoin; RLX, raloxifene; SERM, selective estrogen receptor modulator; SVP, sodium valproate; TGF, transforming growth factor; TNF, tumour necrosis factor; TRAP, tartarate resistant acid phosphatase.

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by renal tubules of the kidneys and slowly by mobilizing calcium from bones thereby contributing to bone loss (Khanna et al., 2009; Valsamis et al., 2006). Though this has been considered to be the primary and the most common mechanism for AEDs-induced bone loss, the same is not always true due to various reasons; firstly, not all patients who develop bony deficits have deficient vitamin D levels (Pack et al., 2011) and secondly, AEDs that are enzyme inhibitors have also been associated with bony adverse effects (Boluk et al., 2004). Thus, other mechanisms such as hypovitaminosis K, calcitonin deficiency, reduced intestinal absorption of calcium, and hyperhomocysteinemia, reported following AED therapy may contribute to adverse effects on bone (Fitzpatrick, 2004; Khanna et al., 2009, 2011).

It is well known that estrogens play an important role in maintaining bone health. Estrogen not only reduces the level of cytokines (IL-6, IL-7, TNF-α) that recruit osteoclasts (Vaananen and Harkonen, 1996) but also opposes the calcium mobilizing actions of parathyroid hormone (Marcus, 1991) and increases the differentiation of osteoblasts mainly through regulation of transforming growth factor, TGFβ3, a bone matrix protein having anti-osteoclastic property (Robinson et al., 1996). The latter plays an important role in controlling bone density by regulating the balance between bone matrix deposition by osteoblasts and its resorption by osteoclasts (Grainger et al., 1999). In addition to this, estrogen deficiency states are known to reduce the deposition of transforming growth factor-β (TGF-β) in rats bone (Finkelman et al., 1992).

We hypothesize here that estrogen deprivation following AED therapy could lead to adverse bony effects. The possible reasons which led us to believe the same are as follows: (a) Many AEDs inhibit human aromatase (CYP19) enzyme (Jacobsen et al., 2008) thereby inhibiting conversion of testosterone to estradiol, (b) AEDs stimulate microsomal catabolism of estradiol and estrone resulting in increased levels of sex hormone binding globulin (SHBG) that in turn lowers testosterone and other adrenal androgens that are aromatized to estrogens (Khanna et al., 2009), (c) AEDs-induced vitamin D deficiency may reduce the expression of aromatase that could lead to bony deficits via (a) described above.

Despite the introduction of second and third generation AEDs in the past decades, phenytoin (PHT) and sodium valproate (SVP) are still considered to be the first line drugs and are widely prescribed in the management of partial seizures and generalized tonic clonic seizures (Nolan et al., 2013). Ample amount of literature is available on the adverse consequences of these AEDs on bone (Boluk et al., 2004; Khanna et al., 2009; Lee et al., 2010; Pack et al., 2011). Calcium–vitamin D (CVD) supplementation is generally recommended on chronic use with these drugs. Although multiple therapy for bone disease such as bisphosphonates, hormone replacement therapy, selective estrogen receptor modulators (SERMs) and calcitonin are approved (Das and Crockett, 2013), very few studies have evaluated the effect of these treatments in AED-induced bone loss. This is surprising as the adverse effects on bone have been reported with these AEDs since 1968 (Kruse, 1968), yet not much research has been carried out with approved anti-osteoporotic agents on AED-induced bony effects until recently where we demonstrated reversal of PHT-induced bone loss in mice by bisphosphonates through reversal of pro-oxidant effects of PHT mediated through PHT-induced hyperhomocysteinemia (Khanna et al., 2011). More recently, the antiepileptic drug and osteoporosis prevention trial (ADOPT) also reported an improvement in BMD in about 70% of epileptic men and prevention of development of vertebral fractures following risedronate with CVD supplementation (Lazzari et al., 2013).

The aim of the present study is to investigate whether raloxifene, a selective estrogen receptor modulator (SERM) and an approved drug for osteoporosis in woman, is effective in preventing or ameliorating PHT and SVP-induced bony deficits in female mice. We selected raloxifene due to multiple reasons: firstly, women may be particularly susceptible to enzyme-inducing AED-induced bone loss because of an independent risk of osteoporosis and fractures that occur in early menopause and in postmenopausal state (Sanders and Geraci, 2013); secondly, most AEDs including PHT and SVP could lead to estrogen deprivation-induced bony deficits via mechanisms described in the preceding paragraphs; and thirdly, raloxifene may increase the threshold for seizures based on some reports on the antiepileptic effects of SERMs in animal models (Borowicz et al., 2002; Scharfman et al., 2009). In the present work, we evaluated the effect of raloxifene (in comparison with calcium and vitamin D3 (CVD) supplementation) on PHT and SVP-induced alterations in bone mineral density and bone turnover markers in Swiss strain albino female mice. Further, the effect of raloxifene on seizures *per se* and on the possible modulating effect on the antiepileptic efficacy of PHT and SVP was also investigated. The probable role of estrogen and TGF-β3 in mediation of bony effects by either AEDs or raloxifene was also explored.

2. Methods

2.1. Experimental animals

Swiss strain female albino mice (25–35 g) raised at the Central Animal House Facility of Jamia Hamdard were used. Prior to the commencement of the experiment, the animals were allowed to acclimate for one week. The animals were housed in polypropylene cages (10/cage) with a room maintained at 23 ± 2 °C and 55 ± 15% humidity with a 12 h light–dark cycle. The mice were fed on a standard pellet diet (Amrut rat and mice feed, Pune, India) and water *ad libitum*. All experiments were performed during the day (between 9:00am and 5:00 pm). The protocol (Project no. 679, year: 2010) was approved by the Institutional Animal Ethics Committee of Hamdard University (Registration No. 173/CPCSEA; January 28, 2000), New Delhi.

2.2. Drugs and chemicals

The following drugs were used: Phenytoin (PHT) (Unicure Laboratories, New Delhi); Sodium Valproate (SVP) (Unicure Laboratories, New Delhi); Raloxifene (RLX) (Dr. Reddy's Laboratories, Hyderabad, India); calcium vitamin D (CVD) (Cipla Pvt. Ltd., Mumbai, India). All other chemicals and reagents were used in this study were of analytical grade.

2.3. Experimental protocol

Preventive treatment. In this experiment, the animals were divided into nine groups of ten animals each and drug was administered orally once daily for a duration of four months: Control group (0.5% CMC, 2 ml/kg); PHT (35 mg/kg); SVP (300 mg/kg); RLX (15 mg/kg); CVD (130 mg/kg + 65 IU); PHT (35 mg/kg) + RLX (15 mg/kg); PHT (35 mg/kg) + CVD (130 mg/kg + 65 IU); SVP (300 mg/kg) + RLX (15 mg/kg); SVP (300 mg/kg) + CVD (130 mg/kg + 65 IU).

Therapeutic treatment. In this experiment, drug was administered orally once daily for a duration of four months followed by one month treatment with bone protective regimen (RLX, Raloxifene; CVDD, Calcium vitamin D + vitamin D): Control group (0.5% CMC, 2 ml/kg); PHT (35 mg/kg); SVP (300 mg/kg); RLX (15 mg/kg); CVDD [CVD (130 mg/kg + 65 IU) + VD (195 IU)]; PHT (35 mg/kg) + RLX (15 mg/kg); PHT (35 mg/kg) + CVDD [CVD (130 mg/kg + 65 IU) + VD (195 IU)]; SVP (300 mg/kg) + RLX