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ORIGINAL ARTICLE

Effect of escitalopram and carbidopa on bone markers in Wistar rats: a preliminary experimental study

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Abstract

In view of the opposite effects of gut and brain serotonin in bone, the key role of Wnt β/catenin pathway in osteoblastic proliferation and the controversial bony effects of selective serotonin reuptake inhibitors antidepressants, the present study investigated the effects of escitalopram alone and in combination with carbidopa (to block gut-derived serotonin) on markers of bone turnover and *Wnt* signaling and micro-CT in male Wistar rats. Escitalopram (2.0 mg/kg, p.o.) and carbidopa (10 mg/kg, p.o.) were administered daily for 40 days following which indicators of reduced (dickkopf-1, sclerostin), and increased (alkaline phosphatase) bone formation and bone resorption markers (receptor activator of nuclear factor κB ligand, tartrate-resistant acid phosphatase 5b) were determined. Our results indicated that escitalopram adversely affected bone as indicated by reduced bone formation and enhanced bone resorption. Further, the effects of escitalopram on bone formation were possibly mediated through gut serotonin while the mechanisms responsible for effects on resorption seem unrelated to gut serotonin. The promising effects of carbidopa on bone formation, as observed in our study, open up exciting possibilities for this drug requiring further investigations.

Keywords Escitalopram · Carbidopa · Alkaline phosphatase · RANKL · Sclerostin

Introduction

Though adverse effects of selective serotonin reuptake inhibitors (SSRIs) on bone health have been studied since 1990, however, results were found to be controversial [1, 2]. While studies demonstrated enhanced bone resorption following SSRIs, they also provided evidence for increased bone formation [3]. It was reported recently that centrally and peripherally synthesized serotonin may have opposite effects on bone formation [4]. Despite this, whether the actions of SSRIs on bone are mediated through brain or periphery is not yet established. Peripheral decarboxylase inhibitor (carbidopa) can inhibit synthesis of serotonin peripherally and thus can be used to study the role of central 5HT modulation by SSRIs on bone. Moreover, carbidopa was recently hypothesized to have protective effects on bone [5], indicating the role of central serotonin in bone formation. Escitalopram is a relatively newer SSRI and is *S*-enantiomer of the racemic citalopram. The drug is comparatively safe and effective in the treatment of depression. However, the preclinical data regarding its effects on bone are still not established.

Recently, activation of the Wnt/β-catenin pathway has been demonstrated to play a key role in controlling proliferation, osteoblast differentiation and, thus, bone formation [6]. Low-density lipoprotein receptor-related protein (LRP5) which acts as a co-receptor in the Wnt/β-catenin pathway is an inhibitor of the enzyme tyrosine hydroxylase (tph), which is the rate-limiting enzyme for serotonin synthesis. Thus, LRP5 inhibits the serotonin synthesis. Dickkopf-1 (DKK-1) and sclerostin (SOST) are two inhibitors of Wnt pathway and can act as biomarkers for reduced bone formation [7, 8].

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Thus, in the present work, an attempt was made to investigate the effect of escitalopram alone and in combination with carbidopa on bone turnover markers including markers of *Wnt* signaling in male rats. Our hypothesis was based on the fact that both gut and brain serotonin have opposite effects on the bone formation and thus a true effect of escitalopram on bone is difficult to ascertain. Therefore, carbidopa, a peripheral decarboxylase inhibitor, blocking the peripheral serotonin synthesis would help ascertain the role of brain serotonin, if any, in mediating the effect of escitalopram on bone.

Materials and methods

Animals

6- to 8-week-old male Wistar rats (150–200 g) were procured from Central animal house facility, Jamia Hamdard, New Delhi. The study was approved by the Institutional Animal Ethics Committee (Project no. 1187, year 2015) and was performed in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Prior to conducting the experiments, animals were acclimatized for 7 days, were housed in polypropylene cages, maintained at controlled conditions of temperature and humidity (25 ± 2 °C, 55–65%) and light dark cycle of 12:12 h and maintained on pellet feed with food and water ad libitum.

Drugs and doses

Escitalopram was obtained from Lupin Ltd., Mumbai, India. Carbidopa was received as gift sample from Shodhana Laboratories, Hyderabad, India. The dose of escitalopram (2.0 mg/kg, p.o.) was derived from corresponding clinical dose shown to produce bone loss in humans [2]. The dose of carbidopa was 10 mg/kg, p.o. [9].

Experimental design

Escitalopram was dissolved in normal saline, whereas carbidopa was dissolved in distilled water [10, 11]. The experimental animals were divided into four groups having six rats in each group. Drugs were administered orally once daily for 40 days. Group 1 served as Normal control group, received normal saline (10 ml/kg, p.o.). Group 2 received escitalopram (2.0 mg/kg, oral). Group 3 was treated with carbidopa (10 mg/kg, p.o.). Group 4 was given combination of escitalopram and carbidopa (2.0 mg/kg, p.o. + 10 mg/kg, p.o.). After 24 h of last treatment, animals were euthanized; blood was withdrawn via cardiac puncture. The femora were dissected out and surrounding muscles and tissues were removed.

Bone tissue sample preparation and assay performed

The femora was weighed individually and homogenized with 10 volumes of phosphate-buffered saline (PBS) at pH 7.2–7.4. The homogenate was centrifuged at 2000–3000 rpm for 20 min. The extraction procedure was repeated twice and aliquots of the bone extracts were used biochemical estimations.

Serum preparation and assay performed

Serum was separated by centrifugation for 10 min at 3000 rpm. Serum samples were stored at − 20 °C until analysis was carried out.

Estimation of DKK-1 and SOST in femora bone

The ELISA kit for the quantification of DKK-1 and sclerostin levels in femora bone was obtained from Kinesis DX (Los Angeles) and Sincere Biotech co., Ltd. (China). The absorbance was measured using ELISA reader at a wavelength of 450 nm [12, 13].

Estimation of alkaline phosphatase (ALP) in femora bone

The concentration of ALP in femoral bone was determined using commercial kit obtained from Span diagnostics (India).

Determination of ALP involves the estimation of protein concentration, which was done by the method of Lowry et al. [14].

Estimation of receptor activator of nuclear factor κB ligand (RANKL) in serum

The estimation of RANKL was done by ELISA kit obtained from Kinesis DX (Los Angeles). The absorbance was measured using ELISA reader at a wavelength of 450 nm [15].

Estimation of tartrate-resistant acid phosphatase 5b (TRAP5b) in femora bone

TRAP5b activity was estimated by the method of Tenniswood et al. [16].

Micro-computed tomography

Lumbar (L2–L4) bone was isolated for analysis. Bone was cleaned off soft tissue, then fixed and stored in

alcohol before scanning. The bone architecture was analyzed using Sky Scan 1076 CT scanner (Aartselaar, Belgium). The lumbar bone was scanned using pixel size of 18 µm with X-Ray source of 70 kV, 100 mA. A total of 178 images of different angular projections were captured. Three-dimensional images were reconstructed using Nrecon software, which simultaneously uses parallel computing of four personal computers, to process the images. Bone mineral density (BMD) and following micro-architectural parameters were recorded: trabecular bone volume (BV/TV, %), trabecular thickness (TB. Th, mm), trabecular separation (TB.Sp, mm), trabecular number (TB.N, 1/mm), trabecular pattern factor (TB. Pf), structure model index (SMI) and connection density (CON. D, 1/mm^3).

Statistical analysis

The data was expressed as mean ± SEM. The results were analyzed by one-way ANOVA followed by Turkey–Kramer Multiple Comparison Test. $p < 0.05$ was considered significant.

Results

Effect of escitalopram, carbidopa and their combination on bone DKK-1 levels

Figure 1a shows the effect of escitalopram, carbidopa and their combination in experimental groups. Escitalopram (2.0 mg/kg, p.o.) administration for 40 days significantly increased femoral DKK-1 levels when compared with

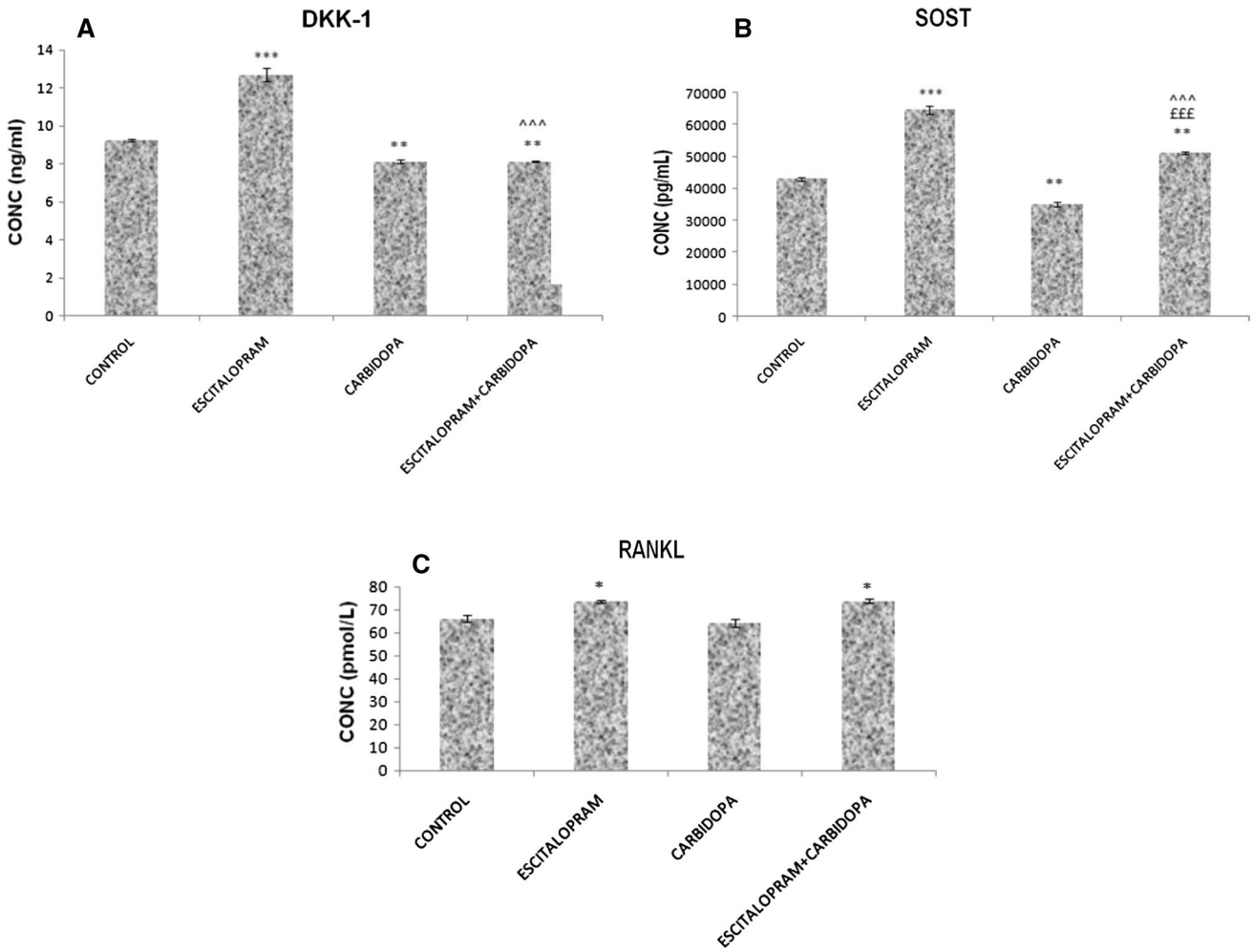

Fig. 1 Effect of escitalopram, carbidopa and their combination on **a** femora bone DKK-1, **b** femora bone sclerostin and **c** serum RANKL levels. Values are represented as mean ± SEM. Group 1: normal control (5.0 ml/kg), Group 2: escitalopram (2.0 mg/kg, p.o.), Group 3: carbidopa (10 mg/kg, p.o.) and Group 4: escitalopram + carbidopa (2.0 mg/kg + 10 mg/kg, p.o.) were administered daily for 40 days. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Group 1, $^{\wedge\wedge\wedge}p < 0.001$ versus Group 2, $^{£££}p < 0.001$ versus Group 3

control group ($p < 0.001$). Co-administration of carbidopa (10 mg/kg, p.o) along with escitalopram (2.0 mg/kg, p.o.) significantly reduced the enhanced bone DKK-1 levels ($p < 0.01$).Carbidopa (10 mg/kg, p.o.) alone for 40 days also significantly reduced bone DKK-1 levels ($p < 0.01$).

Effect of escitalopram, carbidopa and their combination on bone sclerostin levels

The results are presented in Fig. 1b. Escitalopram (2.0 mg/kg, p.o.) administration for 40 days significantly enhanced femoral bone sclerostin levels when compared with control group ($p < 0.001$). Administration of carbidopa (10 mg/kg, p.o.) for 40 days, significantly reduced bone sclerostin levels ($p < 0.01$). While, administration of carbidopa (10 mg/kg, p.o.) with escitalopram (2.0 mg/kg, p.o.) significantly reduced the enhanced bone sclerostin levels ($p < 0.01$).

Effect of escitalopram, carbidopa and their combination on bone ALP levels

The ALP levels in different groups are presented in Table 1. Escitalopram (2.0 mg/kg, p.o.) administration for 40 days significantly reduced bone alkaline phosphatase levels when compared with control group ($p < 0.001$). Addition of carbidopa (10 mg/kg, p.o.) to escitalopram (2.0 mg/kg, p.o.) significantly enhanced the reduced bone alkaline phosphatase levels ($p < 0.05$). Carbidopa (10 mg/kg, p.o.) for 40 days was found to enhance the bone alkaline phosphatase level.

Effect of escitalopram, carbidopa and their combination on serum RANKL levels

The difference in femoral RANKL levels in different groups is summarized in Fig. 1c. Escitalopram (2.0 mg/kg, p.o.) administration for 40 days significantly increased serum RANKL levels when compared with control group ($p < 0.05$). Co-administration of Carbidopa (10 mg/kg, p.o.) with escitalopram (2 mg/kg, p.o.) for 40 days produced results similar to escitalopram alone ($p < 0.05$). Also, carbidopa (10 mg/kg, p.o.) treatment for 40 days did not affect serum RANKL levels ($p > 0.05$).

Effect of escitalopram, carbidopa and their combination on bone TRAP5b levels

The effect of escitalopram, carbidopa and their combination on bone TRAP levels in different groups is shown in Table 1. Escitalopram (2.0 mg/kg, p.o.) administration for 40 days significantly increased bone TRAP5b levels when compared with control group ($p < 0.01$). Carbidopa (10 mg/kg, p.o.) administration failed to reverse increased bone TRAP5b levels, produced by escitalopram (2.0 mg/kg, p.o.) ($p < 0.01$). Carbidopa (10 mg/kg, p.o.) for 40 days did not affect bone TRAP5b levels ($p > 0.05$).

Effect of escitalopram, carbidopa and their combination on lumbar bone micro-computed tomography analysis

The results are presented in Fig. 2a–h. Escitalopram (2.0 mg/kg, p.o.) administration for 40 days resulted in significant reduction in lumbar bone mineral density, reduced BV/TV, TB.Th, TB.N and increased TB.Sp. Carbidopa (10 mg/kg, p.o.) administration did not show any significant change in BMD or bone microarchitecture but it completely reversed the effects of escitalopram on these parameters.

Discussion

In view of the opposite effects of gut- and brain-derived serotonin on bone, and the role of LRP5/Wnt signaling in serotonin synthesis and in controlling osteoblastic differentiation, proliferation and bone formation [17, 18], the present study evaluated the effect of escitalopram, a SSRI, on bone turnover markers in male rats and investigated whether the effects are mediated through brain- and gut-derived

Table 1 Effect of escitalopram, carbidopa and their combination on femora bone TRAP and ALP levels

Experimental group	Dose	TRAP (μmol PNP/h/μg protein)	ALP (μmol PNP/h/μg protein)
Normal control	5 ml/kg	1.03 ± 0.07	34.22 ± 0.52
Escitalopram	2.0 mg/kg	1.35 ± 0.04**	22.52 ± 1.31***
Carbidopa	10 mg/kg	1.13 ± 0.04	39.84 ± 0.37**
Escitalopram + carbidopa	2.0 mg/kg + 10 mg/kg	1.33 ± 0.02**	30.05 ± 0.36*$^{£££,^{^^^}}$

Values are represented as mean ± SEM. Group 1: normal control (5.0 ml/kg), Group 2: escitalopram (2.0 mg/kg, p.o.), Group 3: carbidopa (10 mg/kg, p.o.) and Group 4: escitalopram + carbidopa (2.0 mg/kg + 10 mg/kg, p.o.) were administered daily for 40 days

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus Group 1, $^{^{^^}}p < 0.001$ versus Group 2, $^{£££}p < 0.001$ versus Group 3

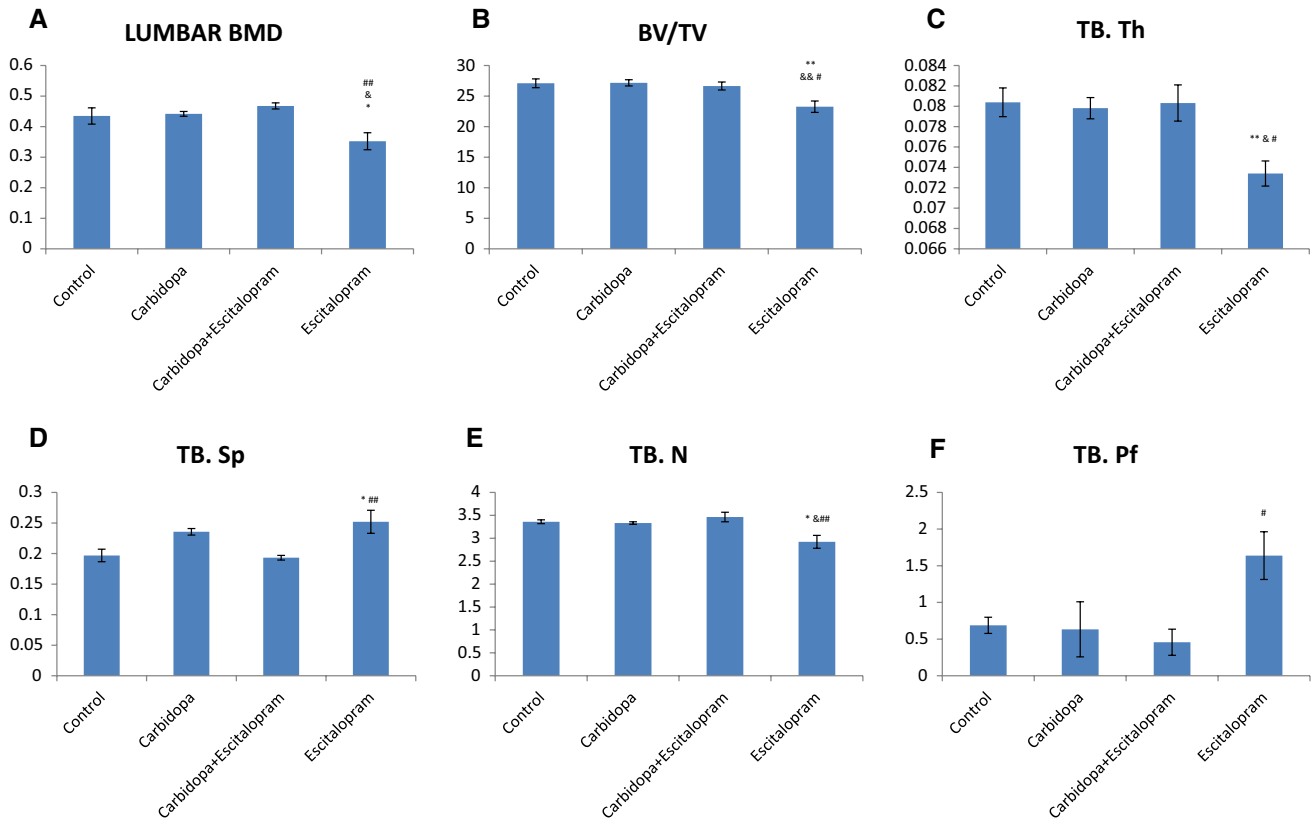

Fig. 2 Effect of escitalopram, carbidopa and their combination on lumbar **a** bone mineral density and **b**–**h** microarchitecture using micro-computed tomography. Values are represented as mean ± SEM. Group 1: normal control (5.0 ml/kg), Group 2: escitalopram (2.0 mg/kg, p.o.), Group 3: carbidopa (10 mg/kg, p.o.) and Group 4: escitalopram + carbidopa (2.0 mg/kg + 10 mg/kg, p.o.) were administered daily for 40 days. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with control, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared with carbidopa, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ when compared with carbidopa + escitalopram

serotonin by giving a peripheral decarboxylase inhibitor carbidopa.

Recent studies have shown a key important role of Wnt/β-catenin pathway in osteoblast proliferation. Binding of *Wnt* protein to the receptor enhances osteoblast proliferation and thus promotes bone formation [19, 20]. Sclerostin and DKK-1 are Wnt pathway inhibitors. The increased level of Wnt inhibitors indicates a negative impact on the bone accrual. We investigated the levels of DKK-1 and sclerostin in femora following escitalopram in view of the fact that LRP5, which acts as a co-receptor in the Wnt signaling pathway is an inhibitor of tph1, which is the rate-limiting enzyme for serotonin synthesis peripherally [17]. Gut-derived serotonin in conjugation with Wnt signaling pathway controls osteoblast proliferation and inhibition of Wnt signaling by the inhibitors depicts reduced bone formation [21]. In our study, oral administration of escitalopram (2.0 mg/kg, p.o.) to male Wistar rats for a period of 40 days was found to enhance bone sclerostin and DKK-1 levels indicating reduced bone formation. The possible mechanism by which gut serotonin is hypothesized to reduce bone formation is via binding to Htr1b receptor, inhibiting the phosphorylation of CREB by PKA, leading to reduced expression of cyclin genes and thus decreasing osteoblastic proliferation [22]. Administration of carbidopa (10 mg/kg, p.o.) with escitalopram significantly reduced the enhanced sclerostin and DKK-1 levels following escitalopram. This indicates that effects of escitalopram on bone formation are possibly mediated via gut serotonin as blocking the peripheral synthesis of serotonin by carbidopa could reverse the effects of escitalopram on bone formation markers. Interestingly, when carbidopa was administered alone, it significantly reduced both sclerostin and DKK-1 levels indicating enhanced bone formation. It is well known that carbidopa reduces peripheral serotonin levels [23, 24]. Our results show that blocking the gut-derived serotonin by carbidopa has positive effects on bone health. However, carbidopa is known to block the peripheral synthesis of not only serotonin but also dopamine. Since the role of dopamine in bone has not been elucidated fully, we hypothesized that it is the reduced serotonin synthesis by carbidopa that is playing the role in the observed effects on bone in our study. Moreover, it was shown recently that dopamine can directly

act on osteoblasts and can enhance osteogenic differentiation and mineralization [25] and hence blocking the peripheral dopamine synthesis may not be responsible for observed effects of carbidopa on bone formation. Our results are also supported by a study on young mice and rats where it was shown that decreasing synthesis of gut serotonin through a small molecule inhibitor of tryptophan hydroxylase 1 could prevent and treat ovariectomy-induced osteoporosis [26]. The positive effects of carbidopa on bone formation are in agreement with the hypothesis of Radaei and Gharibzadeh [5] suggesting the effect of carbidopa in carbidopa–levodopa combination on reducing osteoporotic symptoms in Parkinson's disease patients. This is, to the best of our knowledge, the first study on the role of carbidopa on bone health and thus opens exciting possibilities that needs to be explored further.

Bone-specific isoform of alkaline phosphatase is a glycoprotein that is found on the surface of osteoblasts and reflects the biosynthetic activity of these bone-forming cells and hence bone-specific alkaline phosphatase is considered to be a bone formation marker and its concentration in tissue can be directly correlated with increased bone cell activity [19]. In our study, we measured alkaline phosphatase in femoral bone following antidepressant drug. Treatment with escitalopram for 40 days reduced bone ALP levels again indicating reduced bone formation. Carbidopa reversed the reduced bone ALP levels of escitalopram; thus, gut serotonin could possibly mediate the negative effects of escitalopram on bone formation. As mentioned previously, brain serotonin is reported to enhance (and not reduce) bone formation. Such an enhancement was not observed in our study with any of the three bone formation markers possibly because gut serotonin constitutes about 95% of the total body serotonin synthesis, while brain serotonin constitutes only 5%.

We confirmed the above findings using micro-computed tomography of lumbar bone and observed a significant reduction in bone mineral density and alteration of bone microarchitecture by escitalopram. Further, carbidopa reversed the effects of escitalopram on BMD and microarchitectural parameters confirming our findings on bone formation biomarkers as mentioned above. However, carbidopa per se did not elicit changes in BMD or bone microarchitecture. It may be possible that the biochemical changes are observed early in the investigations while effects on bone microarchitecture may be observed later. Thus, long-term treatment with carbidopa may be necessary to elucidate its positive effects on bone formation markers observed in our study.

Antidepressants are reported to enhance [27] or reduce bone resorption [3]. We evaluated two bone resorption markers following escitalopram treatment in male rats. Receptor activator of nuclear factor-κB ligand (RANKL) is a surface bound molecule found on osteoblasts that serves to activate osteoclast, which are critically involved in bone resorption [28]. Hence, determination of RANKL activity in blood can co-relate with bone resorption. In our study, escitalopram administration for 40 days in rats enhanced serum RANKL activity suggesting enhanced resorption. However, concurrent administration of carbidopa with escitalopram did not alter the effects of escitalopram on RANKL activity. Thus, blocking the peripheral serotonin by carbidopa did not alter the serum RANKL levels. Despite this, it is unlikely that brain serotonin contribute to this effect as Ducy and Karsenty [22] showed that brain serotonin is known to decrease RANKL thereby reducing bone resorption.

Tartrate-resistant acid phosphatase 5b (TRAP5b) activity is regarded as an important cytochemical marker of osteoclast; its concentration in tissue is utilized as a biochemical marker of osteoclast function and degree of bone resorption [29]. In our study, chronic administration of escitalopram enhanced femoral TRAP5b levels. Carbidopa (blocking gut-derived serotonin pathway) was not found to affect the bone TRAP5b levels. Unchanged TRAP5b levels after carbidopa administration suggest that blocking the gut-derived serotonin does not affect bone resorption. Also, blocking the gut-derived serotonin pathway does not alter the enhanced bone resorption marker by antidepressant, signifying the role of pathway other than the central serotonin pathway in the resorption process. Our results on the findings of RANKL and TRAP5b are, however, in contrast with the findings of Battaglino and co-workers [3] who reported that blocking serotonin transporter (5HTT) (as in case of antidepressants) results in inhibition of osteoclast formation as well as reduction in expression of TRAP5b.

To conclude, escitalopram adversely affected bone as indicated by reduced bone formation and enhanced bone resorption marker. The effects on bone formation are possibly mediated through gut (and not brain) serotonin while the mechanisms responsible for effect of these drugs on resorption seem unrelated to gut serotonin. However, this is a preliminary investigation and more rigorous investigations including bone mass parameters and histological evaluation are required to confirm the above findings. The protective effects of carbidopa on bone formation markers observed in this study open up exciting possibilities for further evaluation.

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

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