

Various effects of antidepressant drugs on bone microarchitecture, mechanical properties and bone remodeling

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Abstract

The aim of this study was to evaluate the effects of various drugs which present antidepressant properties: selective serotonin-reuptake inhibitors (SSRIs, fluoxetine), serotonin and noradrenaline-reuptake inhibitors (Desipramine) and phosphodiesterase inhibitors (PDE, rolipram and tofisopam) on bone microarchitecture and biomechanical properties.

Twelve female mice were studied per group starting at an age of 10 weeks. During 4 weeks, they received subcutaneously either placebo or 20 mg kg⁻¹ day⁻¹ of desipramine, fluoxetine or 10 mg kg⁻¹ day⁻¹ of rolipram or tofisopam. Serum Osteocalcin and CTx were evaluated by ELISA. Bone microarchitecture of the distal femur was characterized by X-ray microCT (Skyscan1072). Mechanical properties were assessed by three-point bending test (Instron 4501) and antidepressant efficacy by forced swimming and open field tests.

Fluoxetine displayed lower TbTh (−6.1%, $p < 0.01$) and tofisopam higher TbTh (+5.0%, $p < 0.05$) versus placebo. Rolipram and tofisopam treatments induced higher BV/TV than placebo (+23.8% and +18.3% respectively). Desipramine group had significantly higher cortical area (+4.8%, $p < 0.01$) and fluoxetine lower cortical area (−6.1%, $p < 0.01$) compared to placebo. The stiffness and Young's modulus were lower in the fluoxetine group (77 ± 13 N mm⁻¹, 6431 ± 1182 MPa) than in placebo (101 ± 9 N mm⁻¹, 8441 ± 1180 MPa). Bone markers indicated a significantly higher bone formation in tofisopam (+8.6%) and a lower in fluoxetine (−56.1%) compared to placebo.

These data suggest deleterious effects for SSRIs, both on trabecular and cortical bone and a positive effect of PDE inhibitors on trabecular bone. Furthermore tofisopam anabolic effect in terms of bone markers, suggests a potential therapeutic effect of the PDE inhibitors on bone.

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Keywords: Antidepressant treatment; PDE inhibitor; Selective serotonin-reuptake inhibitors; Bone; Microarchitecture

Introduction

The increased proportions of depressive disorders have multiplied the prescription of antidepressant treatments whatever the countries (Ornstein et al., 2000; van Marwijk et al., 2001). Interestingly in the last 10 years bone researchers dem-

onstrated the importance of the central nervous system in bone metabolism (Takeda et al., 2002; Elefteriou, 2005). Several medications used to treat major depressive disorders have therefore been considered to alter bone properties (Warden et al., 2005).

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter implicated in the etiology of many mental illnesses (Mann, 1999). The serotonin transporter (5-HTT) is the target of a class of antidepressants: the serotonin-selective reuptake inhibitors (SSRI), exemplified by fluoxetine (Prozac®). SSRIs are mainly used to treat depression in adults (Vaswani et al., 2003), as well as children and adolescents (Ryan, 2003). However, serious questions have been raised regarding the influence of SSRIs on

Abbreviations: SSRIs, selective serotonin-reuptake inhibitors; PDE, phosphodiesterase; BV/TV, %, trabecular bone volume; Tb.N, trabecular number; Tb.Sp, μ m, trabecular separation; Tb.Th, μ m, trabecular thickness; DA, degree of anisotropy.

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bone tissue: recent findings of functional serotonergic pathways in bone (Battaglini et al., 2004; Bliziotis et al., 2001) and preliminary clinical evidence demonstrating detrimental effects of SSRIs on the skeleton (Whooley et al., 2004). Warden et al. (2005) demonstrated that bone mineral accrual was impaired in growing mice treated with fluoxetine. Furthermore, 5-HTT null mutant mice had a consistent skeletal phenotype of reduced mass, altered architecture, and inferior mechanical properties (Warden et al., 2005).

Other antidepressants are serotonin and noradrenalin reuptake inhibitors as illustrated by desipramine. Adrenergic receptors have been found in osteoblast and osteoclast (Takeda et al., 2002; Kellenberger et al., 1998). A previous study carried out in our laboratory demonstrated a deleterious effect of a β_2 agonist on the vertebral trabecular architecture in young rats evaluated by microtomography (Bonnet et al., 2005a, 2005b). Our data showed a decrease in BV/TV (-19.7% versus placebo) and biomechanical tests revealed a lower ultimate force (-15.5% versus placebo). In the same way, we have demonstrated in ovariectomized rat a protective effect of a β antagonist (propranolol) on the bone microarchitecture through both an increase in mineralized apposition rate ($+36\%$) and a decrease in osteoclast surface on bone surface (-46%) (Bonnet et al., 2006).

Phosphodiesterases (PDEs) are essential regulators of cyclic nucleotide signaling with diverse physiological functions. Recent advances in molecular pharmacology described PDE isoenzymes as potent biological targets for various therapeutic areas (depression and osteoporosis) (Jeon et al., 2005). Rolipram, a selective inhibitor of PDE4, induced elevation of intracellular cyclic adenosine monophosphate (cAMP) and suppressed expression of proinflammatory cytokines and other mediators of inflammation (Zhu et al., 2001). In rat bone marrow culture, XT-44 (a PDE4 inhibitor) and rolipram stimulated mineralized-nodule formation, whereas it inhibited osteoclast-like cell formation in mouse bone marrow culture (Waki et al., 1999). Interestingly, Waki et al. (1999) observed that Milrinone (a PDE3 inhibitor), and Zaprinast (a PDE5 inhibitor) did not stimulate mineralized-nodule formation as high as Rolipram. Furthermore, a 7.4% femoral BMD increase was observed in OVX rats treated with XT-44 (Waki et al., 1999). Kinoshita et al. (2000) confirmed these results with an increase in femoral and vertebral BMD of 113.2% and 118.2% respectively by Rolipram (20 mg/kg) versus controls.

However, serious issues have been raised regarding the potential use of PDE inhibitor for bone treatment. Although these questions primarily result from suggestions that inhibitors of PDE4 can be emetic (Robichaud et al., 2002), and secondly that the complexity of PDE inhibitors action on bone cells and the lack of in vivo data require further study.

In a drug repositioning strategy, we have identified with SELNERGY (a virtual biological profiling program (Do et al., 2005) an old marketed drug used for central nervous system (CNS) disorders as dual inhibitor of PDE4 and PDE2. This drug named tofisopam belongs to the pharmacological class of benzodiazepine. *S*-tofisopam ($IC_{50}=0.6 \mu\text{M}$) is two-fold more active than the most active isomer of rolipram ($IC_{50}=1.3 \mu\text{M}$)

on PDE4 in the same conditions (Bernard and Lugnier, 2006). The main side effect of rolipram is emesis and mild gastrointestinal distress at doses approaching 15 mg a day, whereas no emetic effects were recorded for *S*-tofisopam. Therefore, tofisopam and its *S*-isomer could be used as a PDE4 and PDE2 inhibitors with a better therapeutic index than rolipram.

To our knowledge there is no specific information on the diverse antidepressant drugs effects (using different pathways of action) on bone microarchitecture and biomechanical properties.

The aim of this work was to investigate the effect of four antidepressant treatments (desipramine, fluoxetine, rolipram and tofisopam) on the structural and mechanical adult bone mice properties.

Materials and methods

Animals and treatment. Sixty female C57BL6J mice (Charles River, France) were acclimatized during two weeks and maintained under constant temperature ($21 \pm 2 \text{ }^\circ\text{C}$) and under 12 h/12 h light-dark cycles during the experience. The mice were housed by five in standard cages and provided with a commercial standard diet. One group of 12 mice, chosen at random, was sacrificed at 12 weeks of age for baseline microarchitecture evaluation. At 12 weeks of age, treatment was initiated with desipramine ($20 \text{ mg kg}^{-1} \text{ day}^{-1}$), fluoxetine ($10 \text{ mg kg}^{-1} \text{ day}^{-1}$), rolipram ($20 \text{ mg kg}^{-1} \text{ day}^{-1}$), tofisopam ($10 \text{ mg kg}^{-1} \text{ day}^{-1}$) and sterile saline (Sigma-Aldrich chimie, St. Quentin Fallavier), injected subcutaneously 5 days per week, during 4 weeks.

Dose and treatment protocol was based upon those described by Waki et al. (1999) and Kinoshita et al. (2000) for rolipram and by Warden et al. (2005) for fluoxetine. The rolipram dose corresponds to a 10-fold higher dose than those typically used to treat depressive symptoms in humans. Dose of tofisopam was determined by its two fold higher activity compared to rolipram and from the therapeutic administration information for its anxiolytic properties (Molcan et al., 1981).

Blood was collected from the intra orbital vessels after an overnight fast at baseline and at the end of the study. After centrifugation, plasma was stored and frozen at $-80 \text{ }^\circ\text{C}$ until analysis. At the end of the study, all groups were sacrificed by an overdose of pentobarbital. In all mice, femurs were excised, cleared of fat and connective tissues. The bones were placed in plastic tubes and frozen at $-20 \text{ }^\circ\text{C}$ for the microarchitectural analysis and biomechanical tests. The procedure for care and killing of animals was in accordance with the European Community standards on the care and use of the laboratory animals (Ministère de l'Agriculture, France).

Forced swimming test. Swimming sessions were conducted by placing mice in individual glass cylinders (46 cm height, 20 cm diameter) containing water at $23\text{--}25 \text{ }^\circ\text{C}$, 20 cm deep, so that mice could not support themselves by touching the bottom with their paws or tail. Swimming sessions were conducted 48 h before death during 6 min. Following swimming session, the mice were removed from the cylinders, dried with paper towels and placed into heated cages for 30 min, and then returned to their home cages. Test sessions were videotaped for later scoring. A single observer, who was blind to the treatment conditions, did all the behavioral scoring.

For each swimming session, the scorer measured the time of the following mice behaviors: (1) immobility-floating in the water without struggling and doing only those movements necessary to keep the head above the water; (2) swimming-showing active swimming motions, more than those necessary to merely keep the head above water, i.e. moving around in the cylinder or diving.

Open field test. The activity of rats was measured in an automated open field ($47 \text{ cm} \times 47 \text{ cm} \times 44 \text{ cm}$) connected to a software-controlled data acquisition device (Actimot Moti 4, TSE Systems, Bad Homburg, Germany). The animals were placed gently in the activity box and the parameters of percentage activity, total distance moved and number of rearing were measured

for 30 min. The tests were carried out 30 min after the administration of the substance.

Morphological and topological characteristics of the trabecular bone.

Trabecular bone microarchitecture of the distal femoral metaphysis was investigated using a microcomputed tomograph (μ CT, Skyscan 1072; Skyscan, Aartselaar, Belgium). The characteristics and methods have already been described elsewhere (Bonnet et al., 2005a, 2005b). The X-ray source was set at 75 kV and 100 μ A, with a pixel size at 6.5 μ m. Four hundred projections were acquired over an angular range of 180° (angular step of 0.45°). The image slices were reconstructed using the cone-beam reconstruction software version 2.6 based on the Feldkamp algorithm. The registered data sets were segmented into binary images. Because of a low noise and the relative good resolution of the data sets, we used simple global thresholding methods. The trabecular bone was extracted by drawing ellipsoid contours with the “CT analyzer” software (Skyscan, Aartselaar, Belgium). Trabecular bone volume (BV/TV, %), trabecular number (Tb.N) and trabecular separation (Tb.Sp, μ m) were calculated by the Mean Intercept Length (MIL) method. Trabecular thickness (Tb.Th, μ m) was calculated according to the method of Hildebrand and Rueggsegger (1997). The structure model index (SMI) was measured for the prevalence of plate-like or rod-like trabecular structures, whereby 0 represents “plates” and 3 “rods” (Hildebrand and Rueggsegger, 1997). The degree of anisotropy (DA) was calculated by superimposing parallel test lines in various directions on the 3D image. DA defines the magnitude of the preferred orientation of the trabeculae. The higher the DA, the more trabeculae are preferentially oriented.

To eliminate the primary spongious, we analyzed 100 slices from the 50 slices under the distal growth plate to the shaft proximally (Fig. 1).

Bone geometric characteristics.

Due to the asymmetric shape of the femoral shaft, 2D bone slice obtained by microcomputed tomography at mid-diaphysis can be characterized by an ellipsoid shape. The large side of the ellipse corresponds to the medial–lateral (ML) direction and the small one corresponds to the anterior–posterior (AP) direction. These two diameters were measured at the mid-diaphysis (=50% of the femur) of the left femur. Cortical width of the long bone is an average of the cortical width measured in ML and AP direction. Cortical width was measured on 5 slices located at 50% of the total length. The results represent an average of those 5 slices (Fig. 1).

Bone mechanical testing.

Four hours before mechanical testing, femurs were thawed at room temperature. The mechanical properties of the femur were determined using a three-point bending test. Each bone was secured on the two lower supports of the anvil of a Universal Testing Machine (Instron 4501, Instron, Canton, MA, USA). The upper roller contacted the femur at the mid-diaphysis with the load direction perpendicular to the medio-lateral diameter. The cross-head speed for all the tests was 0.5 mm/min. Load–displacement curves were generated using specialized software (Instron 4501 software). The following biomechanical characteristics were determined from these curves: ultimate force (maximum force that the bone withstood before fracture); extrinsic energy (energy required to fracture the bone); ultimate displacement (displacement at the ultimate force point); stiffness (extrinsic rigidity of the femur) and Young’s modulus (modulus of elasticity). This method of testing has been previously validated by using Plexiglas standard probes (Turner and Burr, 1993).

The second moment of inertia of cortical bone area was calculated assuming an elliptic cross-section area (Ferretti et al., 1995):

$$I = \frac{\pi}{64} (H^3 B - h^3 b)$$

where H (AP) and h are the external and internal anterior-posterior diameters of mid-shaft respectively, B (ML) and b are the medial-lateral external and internal diameters respectively (Fig. 1).

Biochemical analyses.

Osteocalcin (a marker of bone formation) and C-terminal collagen crosslinks (CTX, a marker of bone resorption) were assayed in duplicate by Enzyme-Linked Immunosorbent Assay (Nordic Bioscience Diagnostics A/S, Herlev Hovedgade, Denmark). The within-assay and between-assay coefficients of variation were less than 10% in our laboratory.

Statistical analysis.

Results are presented as means \pm SEM. A one-way ANOVA test was used to compare the groups for geometric data, architectural parameters, biochemical analyzes, swimming test and open-field parameters. If needed, post hoc differences were determined with the Newman–Keuls test and correlations were performed using the Pearson’s test. A one-way ANOVA with repeated measurements at baseline and 4 weeks were used if necessary. Significance was defined as $p < 0.05$. We did not observe significant difference between groups at baseline for bone markers parameters so we did not mention the data in the Results section.

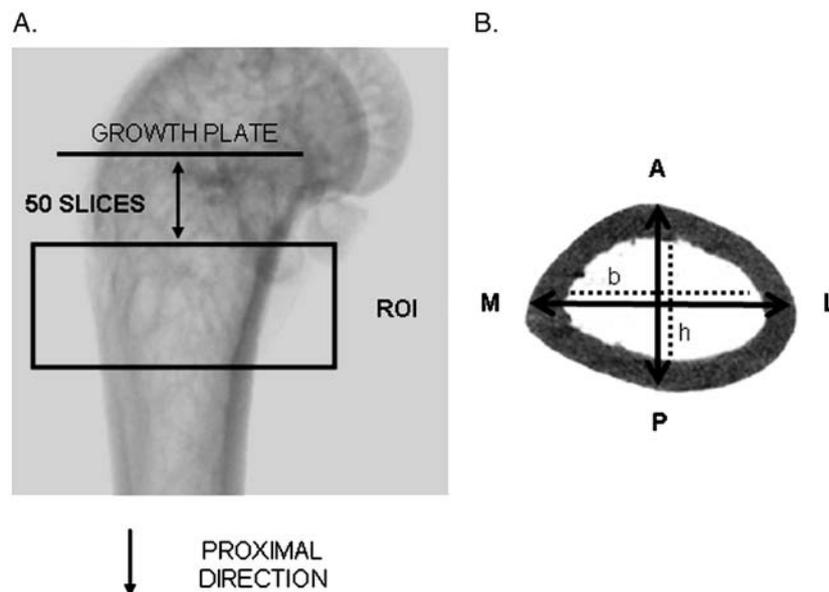


Fig. 1. Radiographic projection of the (A) distal femur and one slice of the (B) mid-shaft cortical acquired by the microcomputed tomography. (A) To eliminate the primary spongious, we analyzed 100 slices from the 50 slices under the distal growth plate to the shaft proximally. Region of interest (ROI) is symbolized by the rectangle. (B) Cortical widths of femur were assessed at the mid-diaphysis (=50% of the femur length). 2D bone slice at mid-diaphysis obtained by microcomputed tomography can be characterized by an ellipsoid shape. An ellipse yields two diameters, (1) where AP and h are the external and internal anterior-posterior diameters of mid-shaft respectively, (2) ML and b are the medial-lateral external and internal diameters respectively.

Results

General observations

For all groups, the body mass of mice increased from baseline to the end of the treatment and we did not observe significant difference in body weight gain. We did not observe difference in food intake between treatment groups measured by analyzing the food weight every day. However, there was a drug influence on activity levels with a lower total distance and number of rearing in rolipram and desipramine groups compared to control and tofisopam groups (Table 1). Swimming test revealed a lower time of immobility in tofisopam, fluoxetine and desipramine compared to controls and rolipram (Fig. 2).

Geometric parameters

We observed a trend to a lower length (−4.5%), bone diameter in ML direction (−4.6%) and cortical area (−6.5%) in the fluoxetine group compared to control group (Table 2). Cortical width in the fluoxetine group was significantly lower than in control group (−11.4%, $p < 0.05$). We did not observe any significant difference in all the geometric parameters measured for the rolipram, tofisopam and desipramine groups compared to control group (Table 2).

Trabecular bone microarchitecture

Distal femur

At the end of the experiment, when compared to the baseline animals, 3D trabecular structure of the control mice revealed a significant gain of trabecular thickness (+8.3%, $p < 0.05$) and a loss of trabecular number (−9.6%, $p = 0.07$). Control animals did not significantly differ in trabecular bone volume fraction compared to baseline.

Trabecular bone volume in rolipram and tofisopam groups is +23.8% and +18.3% higher compared to the control group. We did not observe significant differences between these groups concerning trabecular number (Fig. 3). We observed only a trend to a higher trabecular number in the rolipram group compared to the control group ($p = 0.11$). Trabecular thickness gain was significantly higher in tofisopam group (+13.2%) compared to control group (+8.3%, $p < 0.05$), whereas Tb.Th in the fluoxetine did not significantly increase (+2.2%) during the 4 weeks of treatment. The SMI increase

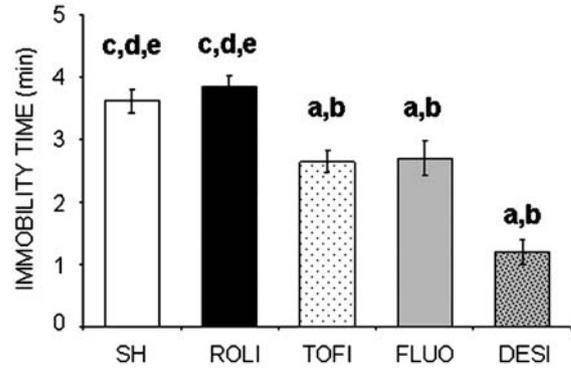


Fig. 2. Evaluation of the anti-anxiety effect of antidepressants by the swimming test in mice. Values represent mean of time (\pm SEM) of immobility behaviors during a 6-min test period ($n = 12$ mice per group). ROLI: rolipram, TOFI: tofisopam, FLUO: fluoxetine, DESI: desipramine. The notes a, b, c, d and e express significantly statistical comparisons between groups. a: compared to CONTROL group ($p < 0.05$), b: compared to ROLI group ($p < 0.05$), c: compared to TOFI ($p < 0.05$), d: compared to FLUO ($p < 0.05$), e: compared to DESI ($p < 0.05$). Means \pm SEM, ML: medio-lateral diameter, AP: antero-posterior diameter.

from baseline was significantly higher in the fluoxetine group (+6.6%, $p < 0.05$) compared to the control group (+1.0%). After 4 weeks of treatment, the SMI of the other groups did not differ from controls. The DA did not significantly differ between groups. The extreme effects of antidepressant treatment compared to controls consisted in a beneficial effect of tofisopam and a deleterious effect of fluoxetine. It has been plotted on Fig. 4.

Biomechanical properties

The bending test revealed a significantly lower stiffness and Young's modulus in the fluoxetine group compared to all the other groups. We noticed a trend to a lower ultimate force in fluoxetine group (−10.5%, $p = 0.10$) (Table 3). There was a significant correlation between stiffness and cortical width ($r = 0.37$, $p < 0.01$) or between Young's modulus and cortical width ($r = 0.29$, $p < 0.01$).

Bone markers

At the end of the experiment, the CTx levels were not significantly different between groups. Osteocalcin level was significantly lower in the fluoxetine group (−56.2%, $p < 0.05$)

Table 1
Influence of antidepressant treatment on the activity measured by openfield

	Control	ROLI	TOFI	FLUO	DESI
Total distance (cm)	5727 \pm 378 ^{b,c}	2849 \pm 279 ^{a,c,d,e}	6231 \pm 309 ^{b,d,e}	4841 \pm 231 ^{b,c}	3568 \pm 286 ^{a,b,c}
Number of rearing	102 \pm 19 ^{b,c}	19 \pm 5 ^{a,c,d,e}	130 \pm 24 ^{b,d,e}	82 \pm 9 ^{b,c,e}	41 \pm 10 ^{a,b,c,d}
Time of rearing (s)	862 \pm 52 ^{b,c}	1320 \pm 45 ^{a,c,d}	798 \pm 43 ^{b,c}	938 \pm 33 ^{b,c}	1241 \pm 51 ^{a,c,d}

ROLI: rolipram, TOFI: tofisopam, FLUO: fluoxetine, DESI: desipramine. The notes a, b, c and d, e express significantly statistical comparisons between groups. a: compared to CONTROL group ($p < 0.05$), b: compared to ROLI group ($p < 0.05$), c: compared to TOFI ($p < 0.05$), d: compared to FLUO ($p < 0.05$), e: compared to DESI ($p < 0.05$). Means \pm SEM.

Table 2
Influence of antidepressant treatment on geometric parameters of the femur

	Control	ROLI	TOFI	FLUO	DESI
Length (mm)	16.27±0.48	15.78±0.45	15.86±0.55	15.54±0.45*	15.85±0.53
ML diameter (mm)	1.94±0.06	1.89±0.07	1.88±0.05	1.85±0.06**	1.89±0.08
AP diameter (mm)	1.25±0.04	1.23±0.03	1.22±0.04	1.18±0.08	1.21±0.06
Cortical area (mm ²)	0.77±0.03	0.78±0.04 ^d	0.79±0.01 ^d	0.72±0.03 ^{b,c,e,*}	0.80±0.04 ^d
Cortical width (mm)	0.35±0.02 ^d	0.33±0.03	0.35±0.02	0.31±0.02 ^a	0.36±0.03

ROLI: rolipram, TOFI: tofisopam, FLUO: fluoxetine, DESI: desipramine. The notes a, b, c, d and e express significantly statistical comparisons between groups. a: compared to CONTROL group ($p < 0.05$), b: compared to ROLI group ($p < 0.05$), c: compared to TOFI ($p < 0.05$), d: compared to FLUO ($p < 0.05$), e: compared to DESI ($p < 0.05$). Means±SD, ML: medio-lateral diameter, AP: antero-posterior diameter. *: Trend of difference versus control ($p = 0.06$); **: Trend of difference versus control ($p = 0.055$).

and higher (+17.9%, $p < 0.05$) in the tofisopam group compared to the control group (Fig. 5).

Discussion

The main findings of this study were that antidepressant treatment effects on the skeleton depend on the mode of action of those substances. Phosphodiesterase inhibitors have beneficial effect on the trabecular bone microarchitecture without significant effect on the bone mechanical properties. In this mode of action, we noted a higher beneficial effect of tofisopam compared to rolipram, suggesting a role of PDE4 but also of PDE2 in the bone metabolism. SSRIs have deleterious effect on bone architecture, microarchitecture and bone mechanical properties suggesting an inhibition of bone growth, despite that we worked on adult mice in the present study. Interestingly we observed that a substance which is both serotonin and nor-adrenaline-reuptake inhibitor (desipramin) did not have the

deleterious effect of SSRI on bone microarchitecture and bone mechanical properties suggesting a beneficial effect of nor-adrenaline-reuptake inhibitors.

The present study confirmed the antidepressant effect of the four different treatments that we tested. First we observed that tofisopam, fluoxetine and desipramine decreased the immobility time in the forced swimming test representing anti-anxiety effect. These results are in accordance with Estrada-Camarena et al. (2004) who demonstrated a significant decrease in immobility and an increase in swimming and climbing. However we did not notice any effect of rolipram. Furthermore, we noticed that opposite to low dose (0.1 mg/kg) (Zhang et al., 2006), high dose of rolipram may decrease the activity (rearing and walking distance) as measured by openfield. According to a human study, antidepressant drug increased the activity of depressive patient (Ernst et al., 2006). It has been suggested that pathophysiology of osteopenia observed in depressive patients includes their lack of activity (Yazici et al., 2003), providing

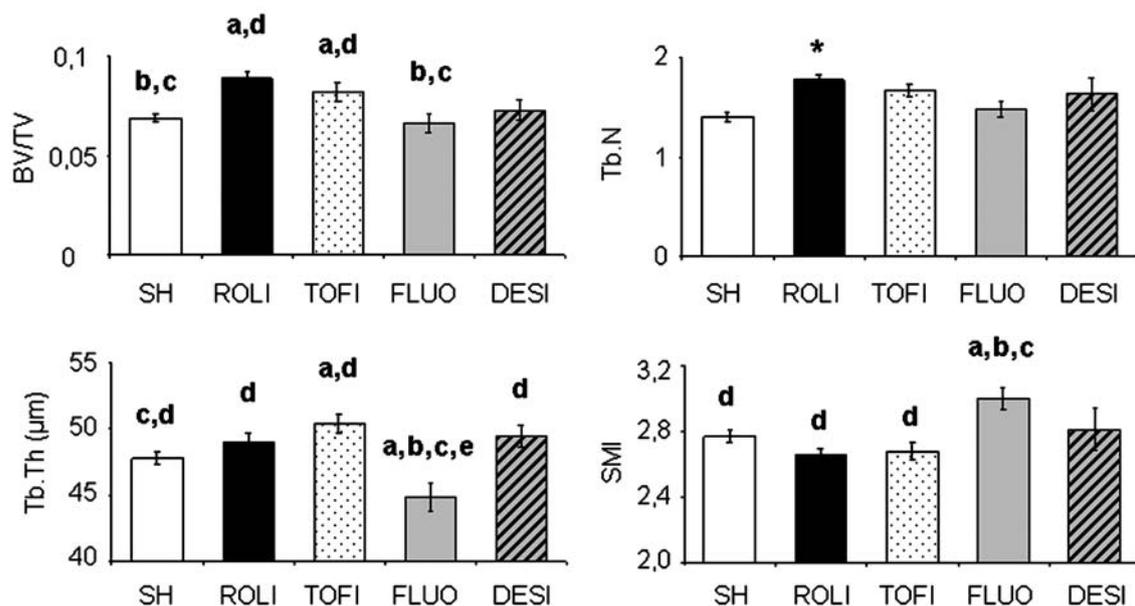


Fig. 3. Effects of antidepressants on trabecular bone microarchitecture of the distal femur after 4 weeks of treatment in mice. ROLI: rolipram, TOFI: tofisopam, FLUO: fluoxetine, DESI: desipramine. Trabecular bone volume (BV/TV), trabecular thickness (Tb.Th, µm), structural model index (SMI, 0: plate to 3: rod). The notes a, b, c, d and e express significantly statistical comparisons between groups. a: compared to CONTROL group ($p < 0.05$), b: compared to ROLI group ($p < 0.05$), c: compared to TOFI ($p < 0.05$), d: compared to FLUO ($p < 0.05$), e: compared to DESI ($p < 0.05$), * represents a trend of difference compared to control group ($p = 0.10$). Means±SEM.

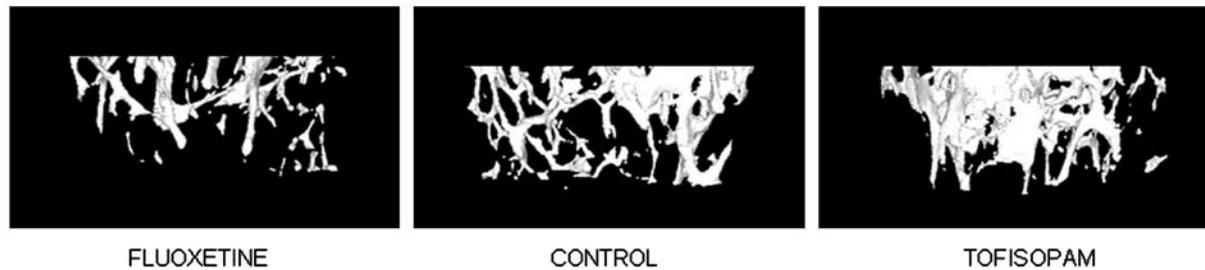


Fig. 4. 3D trabecular bone reconstruction of the distal metaphysis of the femur. We represent bone microarchitecture 3D reconstruction of tofisopam sample which has a beneficial effect and of fluoxetine sample which has a deleterious effect versus control.

further evidence for a functional association between decreased activity and low bone mass (Aparicio et al., 2002).

The skeletal phenotype of our mice treated by fluoxetine is in accordance with the 5-HTT KO mice described by Warden et al. (2005). They observed lower femoral mid-shaft cortical area and ultimate force in 5-HTT^{-/-} compared to 5-HTT^{+/+}. However, despite the lack of significance concerning lower femoral length ($p=0.06$) we believe that the inhibition of 5-HTT decreases the femoral longitudinal bone growth. The biomechanical test used in the present study completed the previous information of Warden et al. (2005). We showed a significant decrease in the stiffness and Young's modulus whereas the ultimate force decrease was not significant. Therefore fluoxetine decreased both the extrinsic and intrinsic stiffness of femurs and tended to decrease the strength of femurs and to make them weaker. Microarchitecture alterations were characterized by a lower trabecular bone volume, trabecular thickness but also by a higher proportion of rod shape indicated by SMI in the fluoxetine group. Bone markers data suggest that fluoxetine decreased bone formation rather than increased bone resorption. This is confirmed by the postulate that lower formation activity would explain the lower trabecular thickness of the microarchitecture whereas a higher resorption activity would induce rather a lower trabecular number. Furthermore, this is in accordance with Warden et al. who showed that a 4-week fluoxetine treatment induced a decrease in endosteal and periosteal bone formation rates at the femoral mid-shaft but also a decrease of the distal metaphysis bone formation rate indicating an alteration of the cortical and trabecular bone (Warden et al., 2005). The 5-HTT has been found to be present in all of the major bone cell types (osteoblasts, osteocytes and osteoclasts). Gustafsson et al. (2006) demonstrated that

fluoxetine effect is due to a direct stimulation of receptor activator of NF- κ B ligand (RANKL) and an inhibition of osteoprotegerin. However, the 5-HTT actions on bone metabolism may also be indirect via other factors such as leptin (Takeda et al., 2002). Surprisingly a recent study found controversial result concerning the impact of fluoxetine on bone microarchitecture, one of the explanations could be the background of their mice which is totally different (Battaglino et al., in press). Particularly, Swiss-webster mice used in Battaglino et al. (in press) study had more trabecular bone (BV/TV $\sim 25\%$) compared to C57BL/6J (BV/TV $\sim 4\%$). Battaglino et al. (in press) suggested that their fluoxetine effects are different from those described by Warden because they worked on adult mice and Warden et al. (2005) on growing animals. But our study contradicted this hypothesis because we described an alteration of microarchitecture by fluoxetine in adult mice. The better explanation for the apparent discrepancy is that the higher fluoxetine treatment duration in Battaglino et al. (in press) study can induce other inhibition than the 5-HTT. It has been shown that fluoxetine can inhibit the membrane currents mediated by activation of various types of neuronal nicotinic acetylcholine receptors (Garcia-Colunga et al., 1997; Maggi et al., 1998).

Interestingly, we did not observe the same skeletal phenotype between fluoxetine and desipramine. Desipramine group did not differ compared to controls. This may suggest that the inhibition of nor-adrenaline transporters would counterbalance the negative bone effect of the serotonin transporters inhibition. At our knowledge no study has described the bone phenotype of nor-adrenaline transporters KO mice. But it is known that nor-adrenalin had a high mitogenic effect on osteoblast by the $\alpha 1$ adrenergic receptor. Furthermore, Yirmiya et al. (2006) recently demonstrated that bone loss induced by depression was

Table 3
Influence of antidepressant treatment on biomechanical properties of the femur

	Control	ROLI	TOFI	FLUO	DESI
Ultimate force (N)	13.37 \pm 0.24	13.23 \pm 0.29	12.90 \pm 0.33	11.96 \pm 0.32*	12.99 \pm 0.30
Displacement (mm ²)	129.62 \pm 2.73	128.91 \pm 2.99	122.88 \pm 4.41	117.09 \pm 3.07	126.67 \pm 3.27
Moment of inertia	0.13 \pm 0.01	0.13 \pm 0.01	0.13 \pm 0.005	0.13 \pm 0.01	0.13 \pm 0.01
Energy (N mm)	6.47 \pm 0.24	6.74 \pm 0.37	6.62 \pm 0.47	6.99 \pm 0.27	8.06 \pm 0.36
Stiffness (N/mm)	100.62 \pm 2.70 ^d	101.90 \pm 2.68 ^d	99.61 \pm 2.45 ^d	77.34 \pm 3.75 ^{a,b,c,e}	95.09 \pm 3.91 ^d
Young modulus (MPa)	8441 \pm 341 ^d	8481 \pm 256 ^d	8019 \pm 167 ^d	6431 \pm 341 ^{a,b,c,e}	7871 \pm 338 ^d

ROLI: rolipram, TOFI: tofisopam, FLUO: fluoxetine, DESI: desipramine. The notes a, b, c, d and e express significantly statistical comparisons between groups. a: compared to CONTROL group ($p<0.05$), b: compared to ROLI group ($p<0.05$), c: compared to TOFI ($p<0.05$), d: compared to FLUO ($p<0.05$), e: compared to DESI ($p<0.05$). Means \pm SD. *: Trend of difference versus control ($p=0.10$).

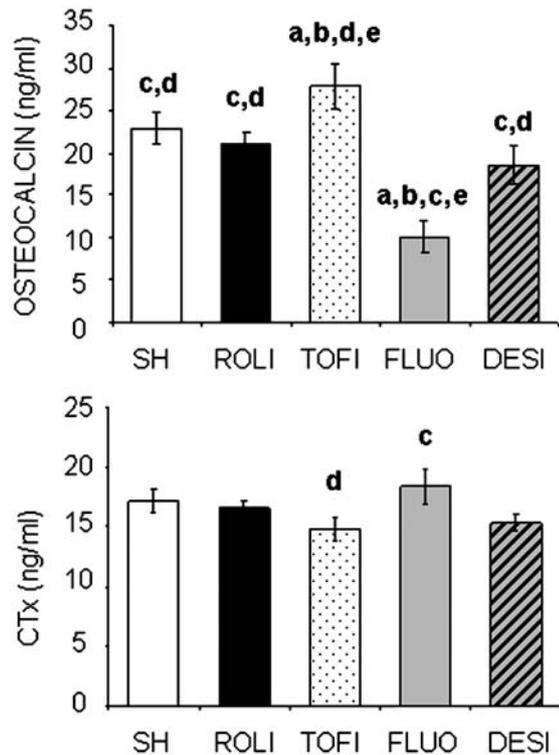


Fig. 5. Effects of antidepressants on bone markers levels after 4 weeks of treatment in mice. Values represent mean of osteocalcin level for bone formation and mean of CTx (C-terminal collagen crosslinks) level for bone resorption. The notes a, b, c, d and e express significantly statistical comparisons between groups. a: compared to CONTROL group ($p < 0.05$), b: compared to ROLI group ($p < 0.05$), c: compared to TOFI ($p < 0.05$), d: compared to FLUO ($p < 0.05$), e: compared to DESI ($p < 0.05$). Means \pm SEM.

inhibited by an imipramine treatment, imipramine being an inhibitor of nor-adrenaline transporters. Genetically modified mice characterized by low sympathetic tone are characterized by an increase in both osteoblast number and activity and a subsequent increase in bone mass (Eleftheriou, 2005). However, the impact of nor-adrenalin on bone seems complex since its effect would depend on the type of receptor (α or β) and on the bone site (Bonnet et al., 2006).

Our study on the effect of PDE inhibitor nuanced the bone microarchitecture effect of rolipram previously described by Waki et al. (1999) or Kinoshita et al. (2000). In our study the rolipram group displayed a higher trabecular bone proportion compared to controls but the increases of trabecular number or thickness were not significant. Furthermore we did not observe significant differences concerning cortical investigation, bone mechanical properties and bone markers.

Nevertheless the results obtained with tofisopam indicated that a combination of PDE4 and PDE2 inhibition is more efficient than a specific PDE4 inhibitor. As previously shown in the in vitro literature, inhibitor of PDE2 can also stimulate bone formation by an increase of BMP (Wakabayashi et al., 2002; Horiuchi et al., 2002). Interestingly we have confirmed by in vitro culture that tofisopam stimulated BMP and particularly BMP2 (data not shown). Recently, Sugama et al. (2006) described the pharmacological basis of the effect of PDE

inhibitors on BMP. They suggested that one possible mechanism is that increased cAMP intensifies BMP signaling by interfering with the negative feedback mechanism of BMP signaling formed by Smad6 induction (Sugama et al., 2006).

Wakabayashi et al. (2002) demonstrated in MC3T3 and ST2 that despite the presence of PDE 1, 2, 3, 4, 7, 8 and 9, only PDE2, PDE3 and PDE4 inhibitors induce an increase of alkaline phosphatase. These data suggest that these compounds (PDE4, PDE3 and PDE2 inhibitors) are potential new candidates for osteoporosis treatment. Although PDE inhibitors were originally thought to stimulate bone formation by potentiation of PGE₂ and PTH, other regulatory factors also appear to be involved, suppression of proinflammatory cytokines known to be associated to bone diseases such as osteoporosis (Zhu et al., 2001).

Our study confirms that PDE inhibitors stimulate bone formation as reflected by an increase of serum osteocalcin levels. Furthermore tofisopam with a two-fold lower concentration has a better effect than rolipram on bone microarchitecture and osteocalcin level. The bone formation effect of tofisopam is of great interest since we know that the major treatments of bone disease are at the present time antiresorbers. However, clinical trials as well as further basic studies will be needed to substantiate the efficacy of PDE inhibitors such as tofisopam for these purposes.

We are aware of some limitations of this study. First we have not compared the antidepressant treatments with classic anti-osteoporotic treatment (such as biphosphonates or PTH) to know if some antidepressants can also protect from bone deterioration as well as anti-osteoporotic treatments. Secondly histomorphometric data would be useful to better understand the effect on bone remodeling, and confirm the bone markers data.

This study highlights the deleterious effect of fluoxetine on bone mechanical properties while treatments like desipramine, rolipram or tofisopam did not modify significantly the bone mechanical properties. This finding is of interest, given the frequent prescription of SSRI to children, adolescents and adults for the treatment of depression and other affective disorders. Furthermore, our study demonstrated that other depression treatment exists with the same efficiency in depression test than fluoxetine without any deleterious effect on bone tissue. Despite the lack of information concerning the precise mechanism of actions of fluoxetine on bone, caution should be taken until further fundamental and clinical data exist.

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