Original Article
A histomorphometric study regarding controversial effects of antidepressants on bone mineral density in menopause

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Abstract: This study investigated the effects of amitriptyline (AMP) and paroxetine (PRO) on bone mineral density and histo-morphometrical structure using an ovariectomized (OVX) rat model. A total of 24 adult female Sprague Dawley rats underwent bilateral ovariectomy 7 days before the experiment. The OVX animals and healthy control rats were divided into four equally sized groups for 4 weeks: a native control group (native), an OVX control group that received distilled water, a group that were administered AMP, and PRO administered group. The bone mineral density (BMD) of the animals' right femurs were determined using a DEXA scan under general anesthesia at two different times: first at the initiation of drug administration after recovery of the OVX rats, and second at the end of drug administration. There was an increase in primary and secondary bone volumes in the AMP group compared with the sham group rats, and there was also a significantly lower volume of primary bone, but a higher volume of secondary bone, in the PRO group compared to AMP group. Osteoporotic histological profiles were shown as dramatic decreases of primary and secondary bone masses, and an increase of bone marrow, especially that containing adipocytes and polymorphonuclear cells, in OVX rats. BMD and risk factors for osteopenia or osteoporosis in postmenopausal women diagnosed with depression or anxiety, and on TCAs or SSRIs, should be assessed before treatment is started, and amitriptyline may be a better choice than paroxetine for maintenance of bone balance.

Keywords: Bone mineral density, depression, menopause, osteoporosis, triyclic antidepressants, selective srotonin reuptake inhibitors

Introduction

Osteoporosis is the most frequently occurring bone remodeling disease, and is characterized by reduced bone mass and the deterioration of bone microarchitecture, leading to an increase in fractures [1]. This serious worldwide health problem particularly affects postmenopausal women, due to acceleration rate of bone resorption associated with reduced level of estrogen [2]. Depression is another major public health problem with substantially higher rates of occurrence in women than in men, and it has been shown that psychological stress due to depression leads to a decrease in osteocalcin levels and a negative impact on bone mineral density (BMD) [3-6]. In addition, two of the most widely prescribed classes of antidepressant drugs, selective serotonin reuptake in-

hibitors (SSRIs) and tricyclic antidepressants (TCAs), have also been associated with a reduction in BMD and a high risk of fracture [7-17]. The majority of studies on this topic have suggested that SSRIs have a negative impact on bone structure, but the extent of the influence of TCAs has remained controversial. Nevertheless, although the mechanism of action remains unclear, the impact of these drugs on bone has shown to depend on dose, exposure duration, exposure time and age of the patient [17-21].

The present study investigated the effects of a TCA, amitriptyline, and an SSRI, paroxetine, on BMD, bone morphometry, and histopathology by using an ovariectomized (OVX) rat model, which is one of the best models for the study of postmenopausal osteoporosis.

Methods

Animals

The animals were housed in facilities accredited by International Guidelines, and the study was approved and conducted in accordance with the Local Ethical Committee for Animal Studies of Ondokuz Mayis University, Samsun, Turkey. A total of 24 adult, female, 3-month-old Sprague Dawley rats weighing 250-300 g were used. The animals were housed individually under standard conditions (12:12 h light-dark cycle at 22 ± 2°C) and nourished with food, as well as water *adlibitum*.

Chemicals

Amitriptyline was obtained from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA), and paroxetine hydrochloride was donated by the Ali Raif Pharmaceutical Company (Levent, Istanbul, Turkey). Both amitriptyline and paroxetine were dissolved in saline at a concentration of 10 mg/1 ml.

Ovariectomy surgery

During the acclimatization period, the rats were fed a diet of standard commercial rat pellets, and 18 rats underwent bilateral ovariectomy 7 days before the experiment. For this procedure, the rats were anesthetized with 100 mg/kg ketamine and 0.75 mg/kg chlorpromazine, which was intraperitoneally (ip) injected. A longitudinal midline incision (0.5-1 cm) was made in the lower abdomen, and the ovaries were removed. After recovery, the OVX animals were randomly divided into four equally sized groups, each consisting of six rats.

Experimental design of animal drug administration

The experimental design of the groups is summarized below. 1. Native group: Healthy control animals that were not subjected to any specific protocol. 2. Sham group: OVX animals that were given 1 mL distilled water ip/daily for 4 weeks. 3. AMP group: OVX animals that were given 10 mg/kg amitriptyline ip/daily for 4 weeks. 4. PRO group: OVX animals that were given 10 mg/kg paroxetine ip/daily for 4 weeks.

Measurement of BMD

BMD (g/cm^2) and bone mineral content (BMC, g) were determined using a DEXA scanner (Hologic QDR 2000, Discovery Series; Hologic, Inc., Waltham, MA, and all animals were placed in a standard supine position for comparison. Data from bilateral femurs were obtained under general anesthesia at two different times: first, at the initiation of drug administration after recovery of the OVX rats (on Day 1), and second, at the end of drug administration (on Day 28). An experimental small animal assessment software was performed. To ensure DEXA functionality, phantom calibration and quality assurance checks were also carried out prior to scanning (**Figure 1**).

Tissue processing for stereological and light microscopic analyses

First, decalcification was performed for the preparation of the hard tissue samples. Specimens were immersed in formic acid, which was changed daily for 2 weeks. After decalcification, femur samples were dehydrated using an ascending series of alcohol (70%-100% ethanol) xylene, embedded in paraffin, and then cut into serial sections of 10 μm in thickness (sagittal plane of the bone specimen) using a Leica RM2125RT microtome (Leica, Wetzlar, Germany). One series of sections was stained with haematoxylin and eosin in preparation for stereological and histopathological examination. All images were photographed by projection to a computer screen via a light microscope with a digital colour camera attachment (Leica DM 7000; Nussloch, Germany).

Stereological analysis

Estimation of femur volume: Femur volume was estimated from microscopic views of the bone samples using a Stereo Investigator System (Stereoinvestigator 9.2, Microbrightfield; Colchester, VT). An appropriate test grid was prepared on this system, and was calculated at test points on a regular grid, which was randomly positioned with the point-counting method. The latter method was applied to each selected section for stereological estimation of the volume of calcified matrix, connective tissue, and newly formed bone. The number of test points hitting the structure of interest was counted for each section. An appropriate coef-
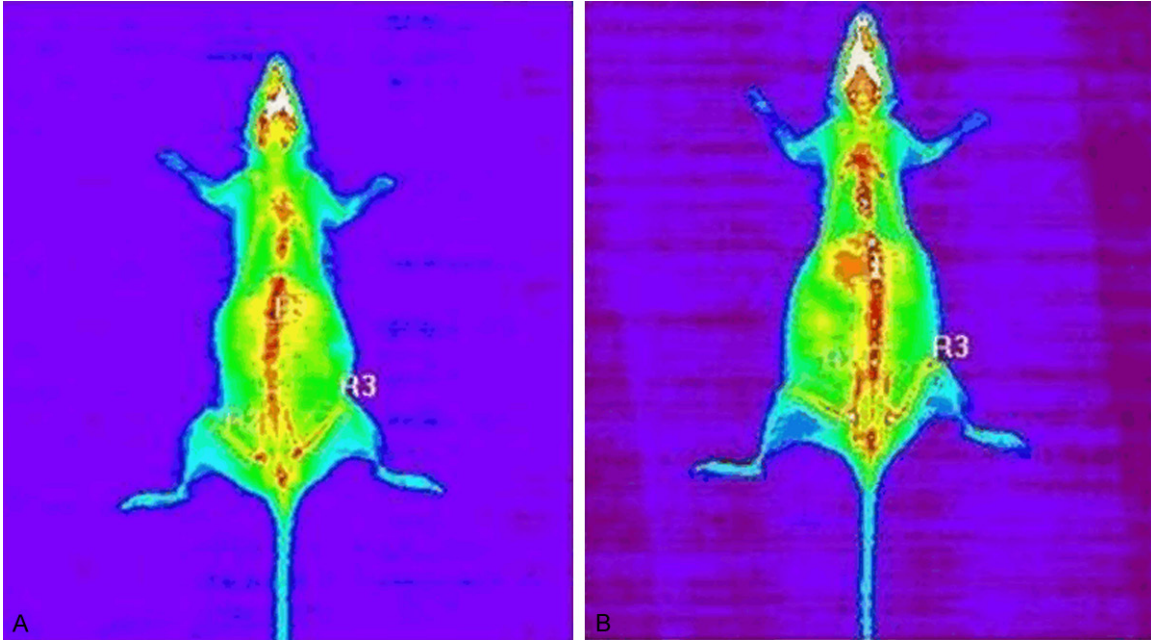
Figure 1. Sample DEXA figures about the Native and Sham groups are seen at (A and B).

Table 1. BMD of the right femurs at the initiation and end of the therapy in the study group

Groups	Day 1 (g/cm^2)	Day 28 (g/cm^2)	Variation
Native	0.163±0.010	0.156±0.017	-0.007±0.009
Sham	0.158±0.019	0.147±0.012	-0.001±0.018
AMP	0.154±0.021	0.164±0.016	0.010±0.022
PRO	0.169±0.025	0.171±0.016	0.002±0.015

PRO: Paroxetine; AMP: Amitriptyline.

ficient error (CE) and a coefficient of variation (CV) were estimated to evaluate the reliability of the grids and sectioning intervals in the areas of interest in the images of the sections [22]. The volumes of all bone sections and other parameters were estimated using the following formula:

$$V = (a/p) \times \sum P \times t$$

As illustrated above, the volume of the structure was estimated by multiplying section thickness (t), total number of points ($\sum P$), and the representation area per point (a/p) in the grid [23, 24].

Estimation of osteoblast/osteocytes number: The boundary of a section that belonged to the dissector pair, called the reference section, was traced with Stereo Investigator software, which was then used to determine the section's cut surface area. The estimated reference sec-

tion area of each femur profile was divided into equal fields in the *x* and *y*-axes of the microscope. Finally, images of all fields in each step, as previously determined via motorized stages of the microscope, were obtained using a charged-coupled device camera. The same procedure was applied to the other section of the section pair, called the look-up section. The adjacent fields were then located on the computer screen, and a suitable unbiased counting frame was manually placed on the kidney photographs with a fixed rule. Finally, the dissector-counting method was applied to these section pairs. Reference and look-up sections were reversed in order to double the number of dissector pairs without obtaining new sections.

The mean number of osteoblast/osteocytes was estimated using the following formula:

Where was the number of counted osteoblast/osteocytes seen in the reference sections, but not in the look-up sections, *t* was the mean section thickness, and *A* was the area of the counting frame.

Statistical analysis

BMD analysis: Data analysis was performed using SPSS for Windows, version 11.5, and the Shapiro Wilk test was used to determine whether or not the distributions of continuous vari-

ables were normal. Homogeneity of variances was analyzed by the Levene test. Data for continuous and intermittent numeric variables were shown as mean ± SD or median (min-max). The significance of differences between the groups was evaluated by one-way analysis of variance (ANOVA). The significance of differences between the groups in terms of preoperative and postoperative measurements was evaluated using a dependent T-test. A *p* value of < 0.05 was considered statistically significant, and the Bonferroni correction test was used in order to correct type 1 errors in multiple comparisons.

Stereological analysis: The data collected were processed by Microsoft® SPSS version 13.0 for Windows software (SPSS, Inc., Chicago, Illinois, USA). Values were expressed in terms of the standard error mean (SEM). One-way ANOVA (i.e., the Bonferroni *posthoc* test) was applied to compare the groups with respect to volumetric data. Values were expressed in terms of the mean and SEM and all statistical values < 0.05 were considered significant.

Results

BMD measurement

BMD measurements of the right femurs were made on the first day and at the end of the paroxetine, amitriptyline, and SF administration in the OVX groups. Both BMD measurements, with a 28-day interval, taken in the native group are shown in **Table 1**. With the exception of the sham group (P < 0.05), no statistically significant difference was determined between the first and second BMD measurements of the groups (P > 0.05). However, the second BMD measurements of the sham group were significantly lower than those of the native group (P < 0.05). The second BMD measurements of the AMP and PRO groups were significantly higher than those of the sham group (P < 0.05). Moreover, BMD values in the PRO group were significantly higher than those of the AMP group (P < 0.05).

Stereological results

The stereological results were provided on the basis of quantitative data (**Figures 2** and **3**). With regard to the volume of the bone marrow, there were significant differences between the groups. In the sham group, the bone marrow volume was significantly increased (P < 0.05, one-way ANOVA). However, in the AMP, and especially in the PRO, group it was significantly decreased (P < 0.05, one-way ANOVA) (**Figure 2**).

The mean volume of the primary bone was decreased in the sham group, compared to that observed in the native group (P < 0.05, one-way ANOVA). The mean volume values of the primary bone in the AMP and PRO groups were lower than those of the native group (P < 0.05, one-way ANOVA). In addition, the mean primary bone volume of the AMP group was higher than that of the PRO group (P < 0.05, one-way ANOVA). However, there were no significant differences among the OVX groups (Sham, AMP, and PRO) in terms of the mean volume of the primary bone (**Figure 2**).

Secondary bone volume was decreased in the sham group compared to the native group (P < 0.05, one-way ANOVA). However, it was increased in the AMP and PRO groups in comparison to the native and sham groups (P < 0.05, one-way ANOVA). In addition, the mean secondary bone volume in the PRO group was higher than that observed in the AMP group (P < 0.05, one-way ANOVA) (**Figure 2**).

The mean number of osteoblasts in the sham group was decreased compared to the native group (P < 0.05, one-way ANOVA), and the mean number of osteoblasts in the AMP group was higher than in the native and sham groups (P < 0.05, one-way ANOVA). The mean number of osteoblasts in the PRO group was higher than in the sham group (P < 0.05, one-way ANOVA). In addition, the mean number of osteoblasts in the AMP group was higher than in the PRO group (P < 0.05, one-way ANOVA) (**Figure 3**).

The mean number of osteocytes in the sham group was decreased compared to the native group (P < 0.05, one-way ANOVA). The values of the mean number of osteocytes in the AMP and PRO groups were higher than those observed in the sham group (P < 0.05, one-way ANOVA), and the mean number of osteocytes in the PRO group was higher in the sham and AMP groups (P < 0.05, one-way ANOVA) (**Figure 3**).

Histopathological results

Routine histological profiles were observed in the histological sections of the femurs of the rats belonging to the native and sham groups,
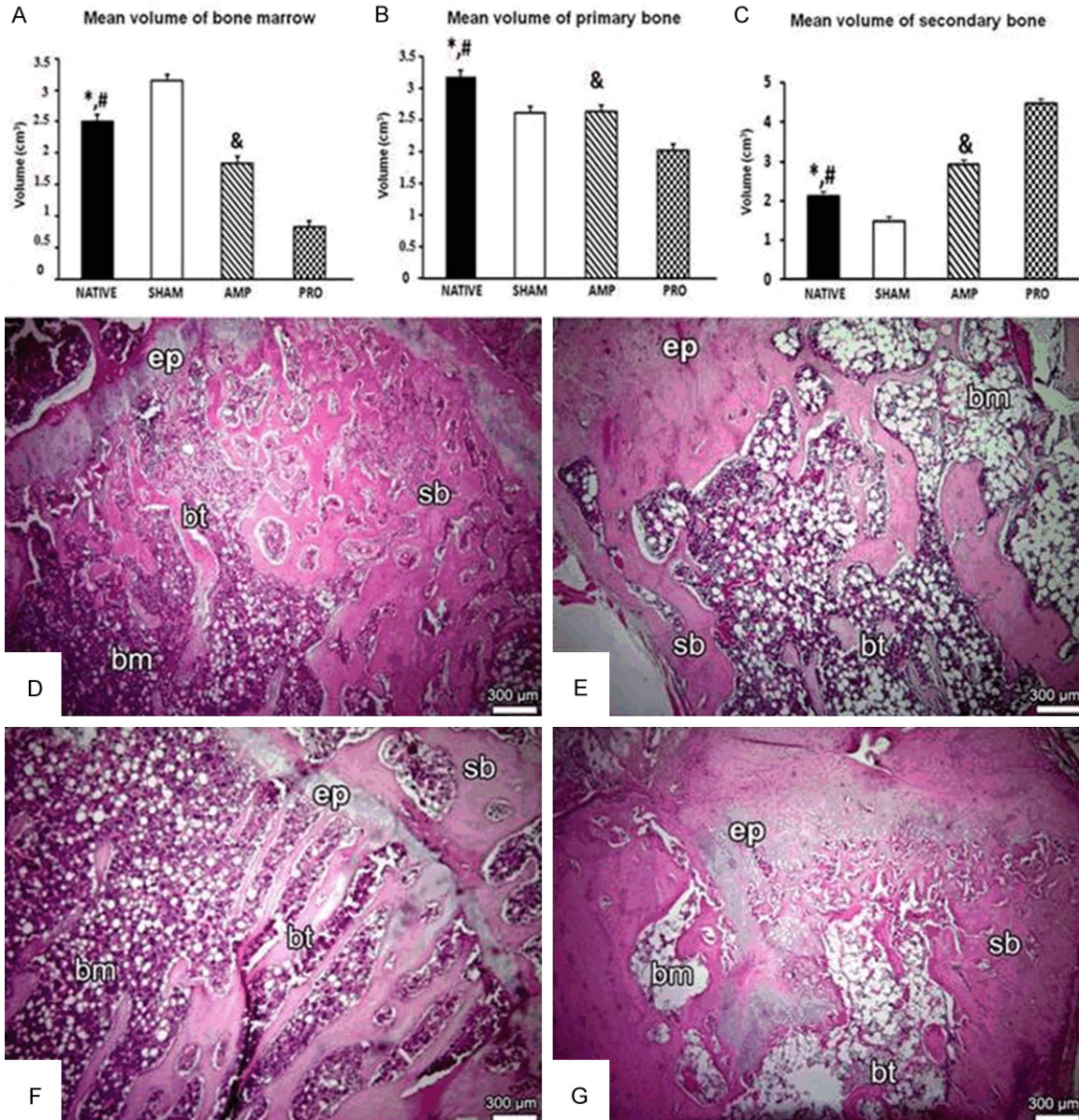
Figure 2. The volumetric results (A-C) and hematoxyline-eosin dyed light micrographes of the right femurs belong to the Native (D), Sham (E) AMP (F) and PRO (G) groups. ep, epiphysis plate; bm, bone marrow; bt, bone trabecules; sb, secondary bone. Signs on the columns in (A-C) shows statistically significant *p* values under the 0.05 level between the Native and Sham (*), Native and AMP/PRO (#), AMP and PRO (&) groups.

and both primary and secondary bone were well-developed. Classical osteoporotic histological profiles were shown in OVX rats as dramatic decreases of primary and secondary bone masses, and increase of bone marrow, especially that containing adipocytes and polymorphonuclear cells. In addition, numerous active osteoclast cells were observed in the sham group. Increases of both primary and secondary bone volumes were detected in AMP group rats compared with sham group rats. In addition, osteoblasts were frequently observed in the AMP group. However, increases of secondary bones were detected in the PRO group compared with the other groups. Osteoclast cells were seen in the PRO group, but some of these were at a distance from the bone areas. Moreover, the Haversian canals in the PRO group were smaller than those observed in the other groups (**Figure 3**).
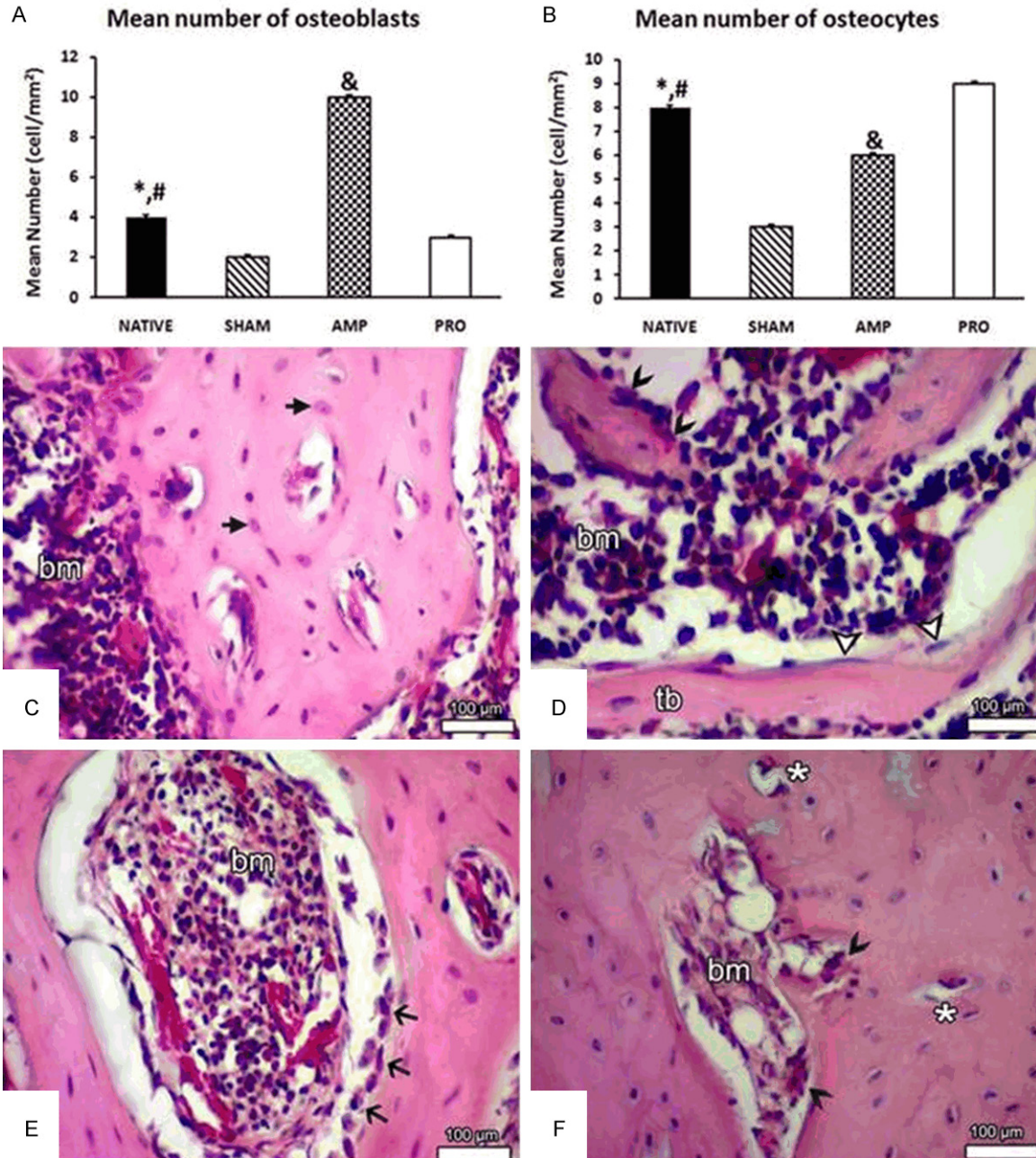
Figure 3. The numerical results about osteoblasts and osteocytes (A, B) and hematoxyline-eosin dyed light micrographes of the right femurs belong to the Native (C), Sham (D) AMP (E) and PRO (F) groups. bm, bone marrow; tb, trabecular bone. Black thick arrows in (A) show healthy osteocytes with euchromatic nuclei; thin arrows in (C) sign active osteoblasts; white (D) and thin black arrowheads (D, F) in (D and F) show inactive osteoblasts and osteoclasts, respectively. Signs on the columns in (A, B) shows statistically significant *P* values under the 0.05 level between the Native and Sham (*), Native and AMP/PRO (#), AMP and PRO (&) groups.

Discussion

Antidepressants are widely used among older adults, worldwide, and it has previously been shown that, in addition to depression *per se*, SSRIs and TCAs also have a direct action on bone metabolism, and are associated with a high risk of fracture [7, 12, 13]. In addition, these associations are consistent after adjustments for confounding variables, such as age, body mass index, and history of fractures. Although the mechanism that is potentially responsible for the affects these drugs exert on the bone remains unclear, several hypotheses

have been suggested. One such hypothesis is that the 5HT2B serotonin receptors aid osteogenic differentiation, and that the serotonin transporter gene has an important role in bone mass, structure, and strength, such that exposure to SSRIs may lead to a decrease in BMC and bone formation [25-27]. However, the results of a number of epidemiologic studies are controversial with regard to the association between TCAs, which have been suggested to contribute to an increased risk of fracture due to adverse anticholinergic effects, including sedation and postural instability, and risk of fracture. Some of these data have indicated a significant association between TCA treatment and an increased fracture risk, whereas no such relationship was found in other studies [7, 19, 20, 28-30].

In the present study, we evaluated the effect of an SSRI (paroxetine) and a TCA (amitriptyline) on bone remodeling using a postmenopausal osteoporosis model in OVX rats. BMD was significantly decreased in the OVX rats at Day 28, compared to the native group. In contrast, it was significantly higher than in the sham group, and there was a significant increase in the PRO group (**Table 1**). Although this finding is consistent with the 5HT2B hypothesis, the additional results of the present study have produced some questions with regard to the effects of paroxetine on bone mineralization.

When comparing stereological analyses of the femur, a significant decrease in mean primary bone volume was detected in both the AMP and PRO groups, whereas the PRO group had a significantly lower mean primary bone volume compared to the sham group (**Figure 2**). Moreover, the mean number of osteoblasts was lower than in the AMP group, but, in contrast, the mean number of osteocytes in the PRO group was higher than in the other study groups (**Figure 3**). These findings are also consistent with both the BMD and histopathological results, suggesting that paroxetine may have a negative effect on the osteoblastic activity of the bone. It appears that the activity of osteoblasts and their conversion to osteocytes could accelerate following paroxetine treatment. In addition, this may be related to the inhibitory activities exerted by osteoblasts on osteoclast cells. At this point, we detected a few osteoclast cells in this group, although some of these

were at a distance from the bone areas, and the Haversian canals in the PRO group were smaller than those observed in the other groups (**Figure 3**).

Thus, the increase in the volume of secondary bone and the reduction in bone marrow and bone cavities caused by paroxetine may have altered bone remodeling and decreased BMD. It may also lead to increase in fragility and fractures, therefore, new insights regarding the molecular pathways of bone remodeling may be required in order to explain the possible mechanisms of osteoporosis and fractures [31, 32].

Since generalized anxiety disorders and depression are widely observed in modern life, and the administration of therapeutic agents has rapidly increased, it is important to ascertain the potential side effects, particularly for elderly people. In the present study, TCAs, which are accepted as safe and effective in the treatment of depression, had a negative impact on BMD as early as the first four months after ovariectomy and a decline in estrogen levels in an animal model that is one of the best menopause models. Therefore, it may be more beneficial to detect the BMD and risk factors for osteopenia or osteoporosis in postmenopausal women diagnosed with depression or anxiety, and on TCAs or SSRIs, before starting treatment.

Disclosure of conflict of interest

None.

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