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Long-Term Fluoxetine Administration Does Not Result in Major Changes in Bone Architecture and Strength in Growing Rats

I. Westbroek,[1,3,4] J.H. Waarsing,[3] J.P.T.M. van Leeuwen,[4] H. Waldum,[2] J.E. Reseland,[5] H. Weinans,[3] U. Syversen,[1,2] and B.I. Gustafsson[1,2]*

[1]Department of Cancer Research and Molecular Medicine, Faculty of Medicine, Norwegian University of Science and Technology, Trondheim, Norway
[2]Department of Internal Medicine, St. Olavs Hospital HF, Trondheim University Hospital, Trondheim, Norway
[3]Department of Orthopaedics, Erasmus Medical Center, Rotterdam, The Netherlands
[4]Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands
[5]Oral Research Laboratory, Faculty of Dentistry, University of Oslo, Oslo, Norway

Abstract Many studies have indicated that serotonin and its transporter play a role in bone metabolism. In this study we investigated the effect of selective serotonin re-uptake inhibitor (SSRI), fluoxetine (Prozac®) on bone architecture and quality in growing female rats. We therefore administrated rats with clinically relevant doses of fluoxetine for a period of 6 months. DXA scans were performed during the treatment period in order to follow parameters as body weight, fat percentage and BMD. After 6 months of treatment, femurs were used to analyze bone architecture and bone strength, by means of μCT scans and three-point bending assays, respectively. We found a slightly diminished bone quality, reflected in a lower bone tissue strength, which was compensated by changes in bone geometry. As leptin and adiponectin could be possible factors in the serotonergic regulation of bone metabolism, we also determined the levels of these factors in plasma samples of all animals. Leptin and adiponectin levels were not different between the control group and fluoxetine-treated group, indicating that these factors were not involved in the observed changes in bone geometry and quality. J. Cell. Biochem. 101: 360–368, 2007. © 2006 Wiley-Liss, Inc.

Key words: fluoxetine; serotonin; BMD; bone architecture; micro-CT; bone quality

The bioamine serotonin (5-hydroxytryptamine; 5-HT), is a well-known neurotransmitter in the central nervous system. In addition, serotonin plays important roles in normal embryogenesis and cell growth, as well as being a regulator of physiological functions such as peristalsis in the gastrointestinal tract and blood pressure regulation [Frishman et al., 1995; Gershon, 1999]. In 2001, we were the first to suggest a relationship between serotonin and bone, by the demonstration of functional receptors for serotonin (5-hydroxytryptamine; 5-HT) in osteoblastic cells [Westbroek et al., 2001]. Furthermore, it has been shown that serotonin does regulate bone cell proliferation, differentiation and activation in vitro [Bliziotes et al., 2001; Westbroek et al., 2001; Gustafsson et al., 2006b]. Moreover, long-term serotonin administration resulted in increased bone mineral density (BMD), stiffer bones, and altered bone architecture in rats [Gustafsson et al., 2006a]. These in vivo serotonergic effects on bone can be direct via serotonin receptors, but may also be indirect via interaction with other bone regulating substances such as leptin and adiponectin [Yamada et al., 1999, 2000; Yamakawa et al., 2003].

Recently, we also demonstrated the expression of the rate-limiting enzyme in serotonin synthesis, tryptophan hydroxylase, in osteo-

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*Correspondence to: Dr. B.I. Gustafsson, Medisinsk avd, gastroseksjon, St. Olavs Hospital HF, N-7006 Trondheim, Norway. E-mail: bjorn.gustafsson@ntnu.no

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blasts and osteoclasts, indicating that bone cells are able to synthesize serotonin [Gustafsson et al., 2006b]. The membrane bound serotonin transporter (5-HTT) expression has also been demonstrated in both osteoblasts and osteoclasts [Bliziotes et al., 2001; Gustafsson et al., 2006b], and is responsible for the cellular internalization of serotonin, and is thus a key protein in serotonergic signaling and serotonin metabolism. This suggests that the serotonin receptor-bearing bone cells are not only able to respond to serotonin, but may also be able to regulate serotonin availability themselves, via its transporter as well as via synthesis.

Interference, with the serotonergic system via 5-HTT may thus affect bone cell function. In a recent study, 5-HTT null mutant mice were shown to have lower BMD compared to their control littermates [Warden et al., 2005]. Furthermore, clinical data already give indications that selective serotonin re-uptake inhibitors (SSRIs), which inhibit 5-HTT action, have an effect on bone metabolism. Moiseiwitsch [2000], has shown that SSRI use by pregnant women affects the craniofacial development of the embryo, while preliminary data show that SSRI use in children may lead to growth retardation [Weintrob et al., 2002]. Battaglino et al. [2004] showed in in vitro experiments that SSRI, fluoxetine (Prozac®), inhibited osteoclast differentiation. On the other hand, in our recent in vitro study, fluoxetine has also been shown to inhibit osteoblast proliferation and decrease the OPG/RANKL ratio in a dose-dependent manner [Gustafsson et al., 2006b]. Interestingly, fluoxetine, has been found not only to inhibit 5-HTT, but has also been found to act directly upon the 5-HT_{2A} and 5-HT_{2C} receptors [Ni and Miledi, 1997; Koch et al., 2002; Palvimaki et al., 1996], and the negative effects of fluoxetine on bone metabolism we found in vitro may, at least in part, be mediated through these receptors [Gustafsson et al., 2006b].

We hypothesize that modulation of the serotonergic system influences bone metabolism, and that long-term use of fluoxetine may have major effects on bone health. In order to investigate this we have administrated clinically relevant doses of fluoxetine to growing (but sexually mature) rats for 6 months and studied BMD, bone architecture, and bone mechanical properties. As some studies suggest that antidepressants in humans also affect body weight and energy expenditure [Dryden et al., 1996; Fava et al., 2000; Moosa et al., 2003] we also investigated whether fluoxetine treatment significantly altered leptin and adiponectin plasma concentrations in these animals, as these are also very important factors in bone metabolism.

MATERIALS AND METHODS

Animals

Twenty-one, 8 weeks old Sprague-Dawley female rats (±200 g) were housed solely in wire-top cages with aspen woodchip bedding from B&K Universal Ltd. Room temperature was $24 \pm 1^{\circ}C$ with a relative humidity of 40–50% and a 12-h light/dark cycle. The Rat and Mouse Diet of B&K and tap water were provided ad libitum. Fluoxetine was obtained from Eli Lilly Norge A.S. (Oslo, Norway). Eleven rats were given fluoxetine (5 mg/kg, each dose dissolved in 1 ml fresh tap water) once daily, via gastric intubation; ten controls received tap water, also via gastric intubation. The fluoxetine dose used, was determined by titration to serum levels of fluoxetine between 1 and 2 μM. Dual X-ray absorptiometry (DXA) measurements were performed at the beginning and after 4 and 6 months of treatment. After 6 months the animals were euthanized. Blood and both femurs were collected for further analysis. Before all procedures (except for gastric intubation), the animals were anesthetized with 2 ml/kg body weight of a combination of haldol (1.65 mg/ml), fentanyl (0.25 mg/ml), and midazolam (2.5 mg/ml). The Animal Welfare Committee at Trondheim University Hospital approved this study.

Dual X-ray Absorptiometry (DXA) Measurements In Vivo

The body weight (g), lean body mass (g), fat content (as percentage of body weight), and femur and total body BMD (g/cm^2) were measured in anesthetized animals by means of DXA, using a Hologic QDR 4500A with special small animal software. Measurements were performed in duplicate at the start, and after 4 and 6 months of treatment. One female Sprague-Dawley rat was analyzed in the DXA scanner, ten times with repositioning, in order to determine the coefficient of variance (CV). The CV was expressed as the percentage of standard deviation (SD) of the mean. For body weight the CV was 0.063%, for lean body mass

0.35%, for fat content 3.16%, for femur BMD 1.29%, and for total body BMD it was 0.68%.

Leptin and Adiponectin Plasma Assays

Concentration of leptin and adiponectin in plasma was measured by competitive radioimmunoassays according to manufacturer's instructions (Linco Research, St. Charles, MO). Samples were diluted 1:500 prior to adiponectin analysis. Intra-assay variation was 1% for the adiponectin kit and 1.7% for the leptin kit.

Bone Architecture

After 6 months treatment, right femurs were dissected from euthanized animals and stored in 4% formalin solution until further use. Bone architecture was studied using micro-computed tomography (μCT) scanning. The proximal femur, including the femoral head and the metaphysis (Fig. 3A) of the dissected femurs were scanned in a SkyScan 1072 microtomograph (SkyScan, Antwerp, Belgium), with a voxelsize of 11.89 μm. Scans were processed, and three-dimensional morphometric analyses of the femurs were done using free software of the 3D-Calculator Project (http://www.eur.nl/fgg/orthopaedics/Downloads.html). The datasets were separated in a femoral head-part and metaphysis-part (Fig. 3A). Cortical volume, cortical thickness, trabecular bone volume, endocortical bone volume (including trabeculae), trabecular bone volume fraction (as a fraction of endocortical bone volume), trabecular thickness [Hildebrand and Ruegsegger, 1997b], connectivity density [Odgaard and Gundersen, 1993], and structure model index [Hildebrand and Ruegsegger, 1997a] were determined. Cross-sectional polar moment of inertia (MOI_z) was determined over the complete dataset (femoral head + metaphysis). The mean of the periosteal perimeter was calculated for the metaphysis-dataset.

Mechanical Properties (Three-Point Bending)

After 6 months treatment, left femurs were dissected from euthanized animals and were stored in PBS at −20°C until further use. In order to determine cross-sectional MOI_x with respect to the neutral axis (x) (Fig. 4B) at break-section, the entire bones were first scanned in a SkyScan 1072 microtomograph (SkyScan), with a voxelsize of 18 μm. Femurs were thawed and equilibrated to room temperature before three-point bending experiments, and soft tissue was removed. Individual femurs were then placed in the custom made three-point bending device, while condyles were positioned at 0 mm, as shown in Figure 4A. Just above the condyles, the femur was placed on the left load post, while the *trochanter tertius* was placed on the right load post (Fig. 4A) and steady positioning was assured. The distance between the two loading posts was ≥15 mm, which guarantees that 85–90% of the flexture of the bone is due to bending [Turner and Burr, 1993]. The femurs were kept as moist as possible during the three-point bending (Single Column Lloyd LRX Testing System, Lloyd Instruments, Fareham, UK). In order to guarantee reproducible results and steady positioning, five cycles of pre-conditioning were performed with a displacement rate of 0.01 mm/s, a 4 N limit and a hold-time of 1 s, after which the test system returned to a position where a load of 0 N was achieved. Immediately after pre-conditioning, the mechanical test was started, with a displacement rate of 0.01 mm/s, a sampling rate of 20 Hz, and a limit of 1.5 mm displacement, a displacement at which all femurs were certain to have been broken. Displacement (mm) and force (N) were registered, and displacement to force graphs were made. Maximum force (N) was determined, and energy absorption (mJ), strain (%), ultimate stress (N/mm^2), and Young's modulus (N/mm^2) were calculated according to Turner and Burr [1993]. The two latter parameters represent bone tissue properties and thus required MOI_x at break-section. In order to determine MOI_x at the break-section, the location of the break-section was determined, after the femur was broken. Using length L depicted in Figure 4A, we could calculate which cross-section of the μCT scans had to be used to calculate MOI_x. Furthermore, in order to calculate the MOI_x with respect to the neutral axis (Fig. 4B) we used the *trochanter tertius* in the scans as a reference for orientation.

Bone Histomorphometry

Fixed femurs were embedded in polymethylmethacrylate (PMMA). Of each femur sequential longitudinal of 6 μm sections were made. In order to investigate bone resorption, Tartrate-Resistent Acid Phosphatase (TRAP) staining was performed on eight sequential sections to stain osteoclasts, as described [Cole and Walters, 1987]. In order to visualize unminer-

alized matrix a Goldner staining was performed on another eight sequential sections.

Statistics

Statistical analyses were performed in SPSS 12. Normality of the parameters was tested by means of Shapiro-Wilk, and normality was assumed at P values above 0.05. To test for differences paired t tests were done to analyze normally distributed datasets, while Mann–Whitney U tests were performed on the non-normally distributed datasets. The parameters obtained at different time points by means of DXA were analyzed by using a Kruskal-Wallis test to test for differences between the groups. General Linear Model tests were used to test for interactions between time and treatment in the DXA data. Significant differences/interactions were assumed at P values below 0.05.

RESULTS

Bone Mineral Density, Fat Percentage, and Leptin and Adiponectin Plasma Levels

Animals that received fluoxetine did not significant differ compared to control animals in any of the parameters determined by DXA (Fig. 1). All parameters were significantly affected by age during the whole experiment, indicating that growth of the animals had an influence on all parameters. However, no significant interaction between age and fluoxetine treatment was found, which indicates that fluoxetine treatment did not affect the change in

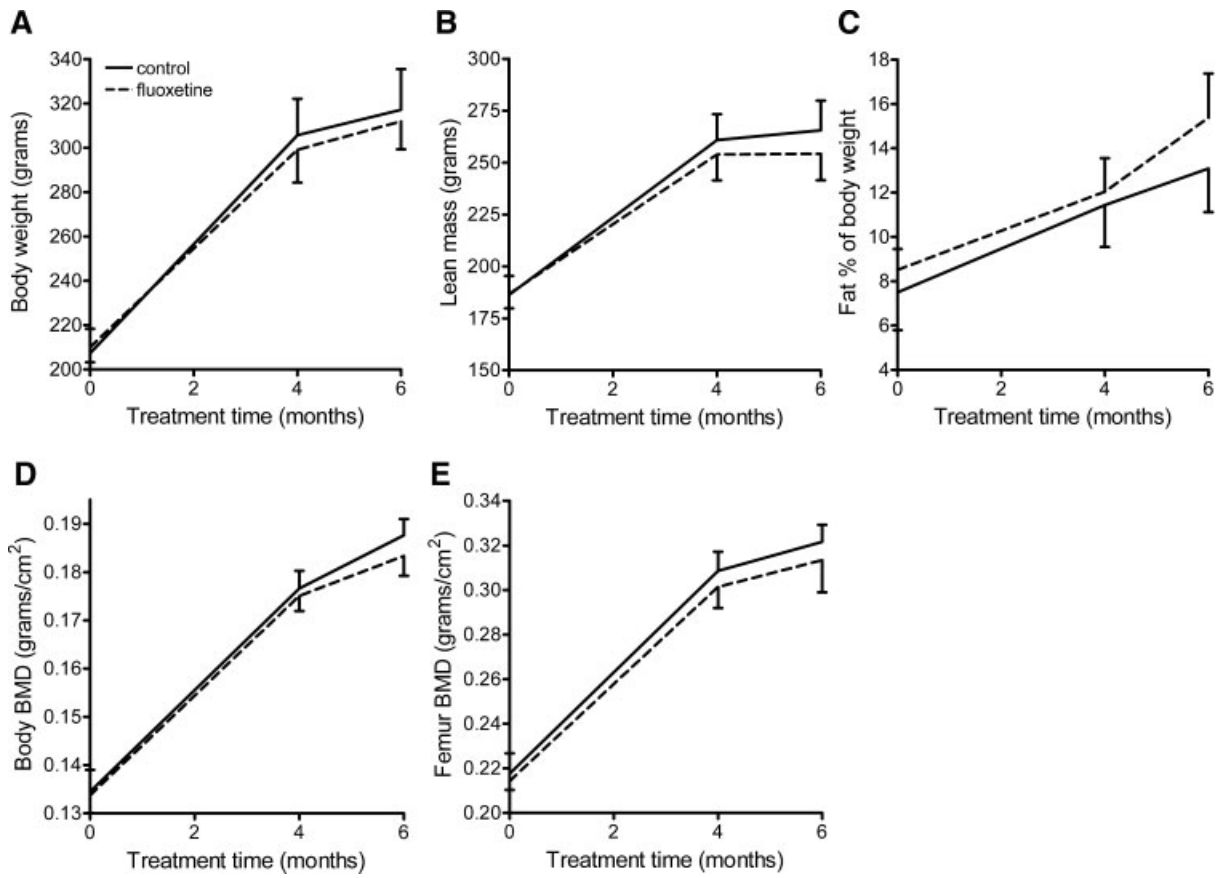

Fig. 1. In vivo DXA measurements at beginning and after 4 and 6 months of treatment with fluoxetine. **A**: The body weight of fluoxetine-treated animals is not significantly different from control animals. **B**: The lean body mass of fluoxetine-treated animals does not significantly differ from control animals. **C**: Fat content (as percentage of body weight) was higher at all time points in the fluoxetine-treated animals, but this never reached significance. **D**: Body BMD did not differ significantly between the two groups. **E**: Differences in femur BMD between the two groups almost reached significance (Kruskal Wallis $P = 0.078$). All determined parameters showed a significant time effect, indicating that all parameters were subjected to growth. There was no interaction between time and fluoxetine treatment, suggesting that fluoxetine treatment did not affect growth. Results are given as means $\pm$ 95%-CI, $n_{control} = 10$ and $n_{fluox} = 11$.

the parameters due to growth. Femoral length also did not differ significantly between the two groups, also indicating that fluoxetine did not affect growth (data not shown). Femoral BMD was slightly lower in the fluoxetine-treated animals compared to control animals and nearly reached significance ($P = 0.078$) (Fig. 1E).

The fat percentage seemed higher at all time points in the fluoxetine-treated animals, however, statistical analyses pointed out that fluoxetine treatment did not significantly affect fat percentage (Fig. 1C). As anti-depressants are known to affect body weight and energy expenditure in humans [Dryden et al., 1996; Fava et al., 2000; Moosa et al., 2003], we also determined circulating leptin and adiponectin levels in these animals. Both leptin (Fig. 2A) and adiponectin (Fig. 2B) levels were not significantly different between control animals and fluoxetine-treated animals.

Bone Architecture, Mechanical Properties and Histomorphometry

In the metaphysis part of the femur (Fig. 3A), fluoxetine treatment resulted in a lower trabecular thickness ($P = 0.033$; Fig. 3B) and higher endocortical bone volume ($P = 0.043$; Fig. 3C). We observed a trend towards an increase in perimeter in the fluoxetine-treated animals, however, this failed to reach significance ($P = 0.199$; Fig. 3D). All other parameters that were determined in μCT analysis did not show significant changes after fluoxetine treatment (Table I).

Three-point bending experiments were performed in order to investigate mechanical properties of the femurs. Maximum force and strain were not significantly different in the fluoxetine group versus the control group (Fig. 4D,E). Both ultimate stress (Fig. 4F) and Young's modulus (Fig. 4G) were significantly decreased in the fluoxetine group, meaning less force per area was needed to deform and ultimately break the bone tissue. This weakness in bone tissue strength in the fluoxetine group seemed to be opposed by a slightly higher ($P = 0.075$) cross-sectional MOI_x at break-section (Fig. 4C), eventually leading to similar bone strength as the control animals (maximum force did not differ).

All sections studied for TRAP staining were found to be negative (data not shown), while a positive control (rat femur) did show positive osteoclasts. Osteoclast activity may be very low in rats of this age, since bone turnover may already be low. Furthermore, no positive staining for unmineralized matrix was found in the sections (data not shown), which may also be due to a low bone turnover state or due to the fact that mineralization is known to occur very quickly in rodents.

DISCUSSION

We have previously demonstrated that long-term serotonin administration to female rats leads to higher BMD [Gustafsson et al., 2006a]. Here we present, to our knowledge, the first study on long-term effects of SSRI, fluoxetine, on bone in rats. In this study, we show that fluoxetine treatment, in clinically relevant doses, does not have major effects on bone

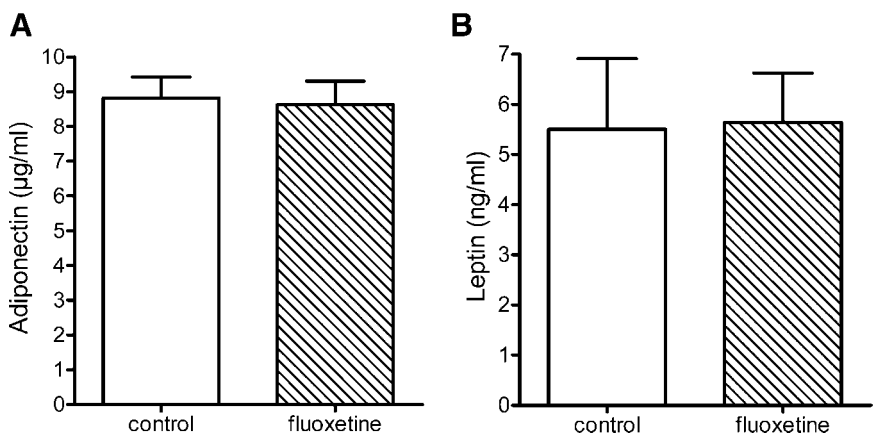

Fig. 2. **A**: Adiponectin levels in plasma did not differ between fluoxetine-treated animals and controls. **B**: Leptin levels measured in plasma were not different between fluoxetine-treated animals and control animals. Results are given as means + 95%-CI, $n_{control} = 10$ and $n_{fluox} = 11$.

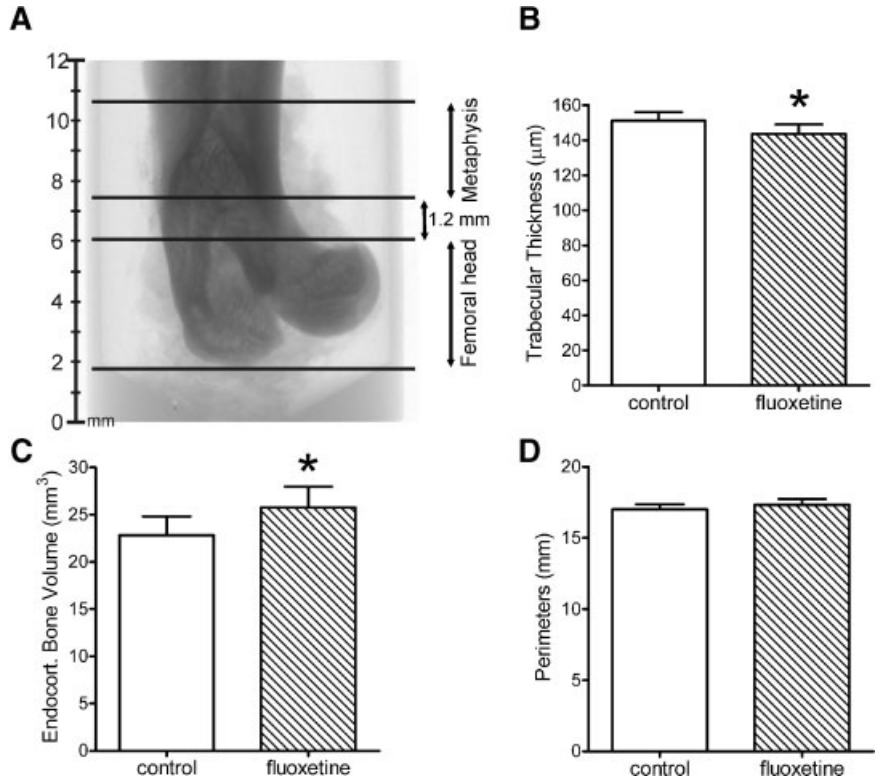

Fig. 3. **A**: Typical μCT scan of rat femur with a voxelsize of 11.89 μm. Metaphysis-dataset and femoral head-dataset are depicted. **B**: Trabecular thickness in the metaphysis is shown and is found to be significantly lower in fluoxetine-treated animals versus control animals (*t*-test $P = 0.033$). **C**: Endocortical bone volume in the metaphysis was significantly higher in fluoxetine-treated animals compared to the control animals (*t*-test $P = 0.043$). **D**: Perimeters in the metaphysis were not significantly different between the two groups. Results are given as means + 95%-CI, $n_{control} = 10$ and $n_{fluox} = 11$.

TABLE I. Mean Values ± SEM of all Parameters Determined by Means of μCT Scanning Analysis

	Control (n = 10)	Fluoxetine (n = 11)	*P*-value
Metaphysis			
Cortical volume (mm^3)	29.63 ± 0.51	28.93 ± 0.46	0.329
Cortical thickness (μm)	772.88 ± 16.43	735.45 ± 16.43	0.094
Trabecular bone volume (mm^3)	6.14 ± 0.33	6.34 ± 0.30	0.314
Endocortical bone volume (mm^3)	22.82 ± 0.89	25.75 ± 1.00	0.043
Trabecular bone volume fraction	0.27 ± 0.007	0.24 ± 0.01	0.096
Trabecular thickness (μm)	151.09 ± 2.13	143.64 ± 2.42	0.033
Connectivity density	15.47 ± 1.86	16.77 ± 0.98	0.307
Structure model index	0.69 ± 0.05	0.71 ± 0.07	0.798
Perimeter (mm)	17.00 ± 0.16	17.32 ± 0.19	0.055
Femoral head			
Cortical volume (mm^3)	23.61 ± 0.78	23.23 ± 0.72	0.729
Cortical thickness (μm)	456.19 ± 7.42	445.50 ± 9.97	0.408
Trabecular bone volume (mm^3)	9.55 ± 0.61	10.17 ± 0.41	0.605
Endocortical bone volume (mm^3)	18.06 ± 0.97	19.87 ± 0.88	0.182
Trabecular bone volume fraction	0.526 ± 0.011	0.513 ± 0.008	0.152
Trabecular thickness (μm)	161.89 ± 3.17	159.60 ± 2.17	0.552
Connectivity density	54.50 ± 5.27	49.39 ± 1.78	0.352
Structure model index	0.46 ± 0.09	0.30 ± 0.09	0.242

In the metaphysis-dataset, endocortical bone volume as well as trabecular thickness were significantly different. Graphs of important parameters are given in Figure 3.

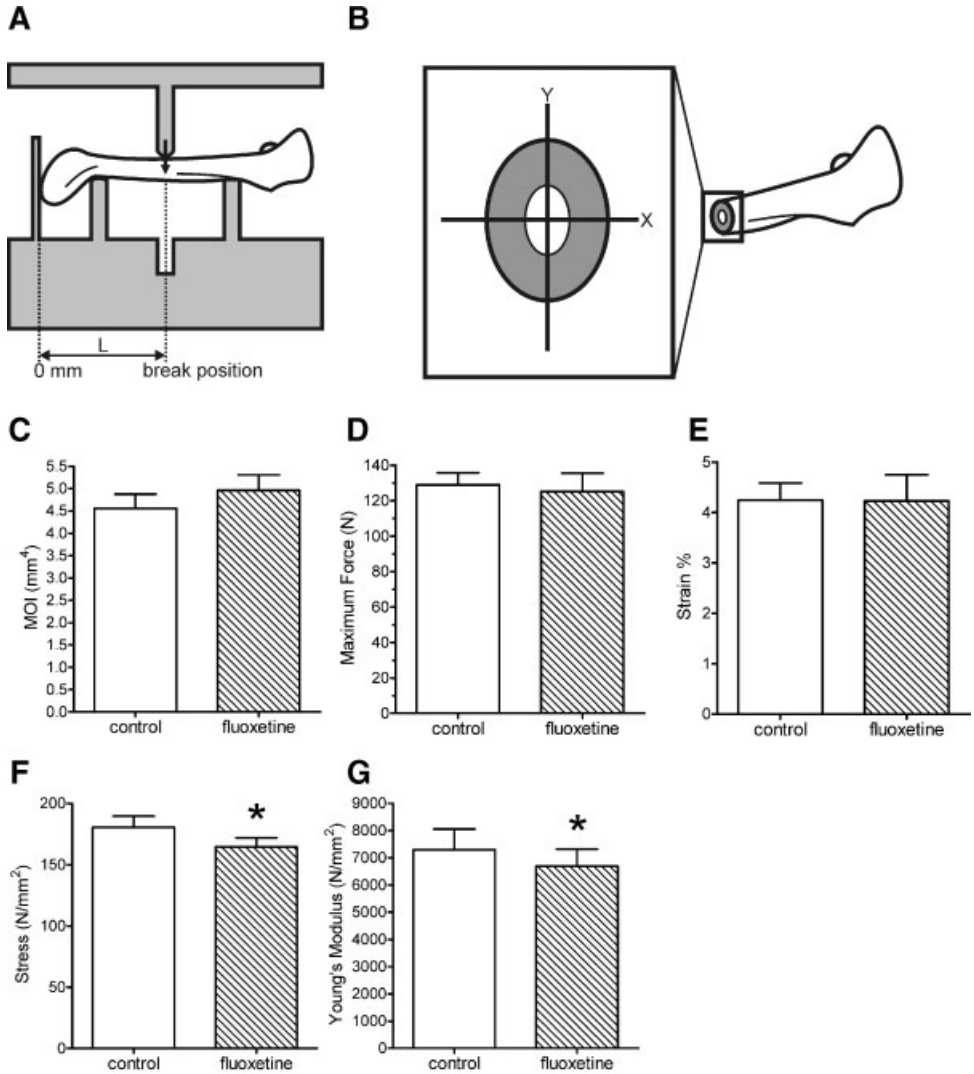

Fig. 4. **A**: Custom made three-point bending loading device. The femur is positioned for mechanical test experiment as described in the Materials and Methods section. The distance L depicts the distance between 0 mm position where the condyles are set at, and the break position. **B**: Schematic view of the break-section of the femur after three-point bending. The neutral axis (x) is depicted. **C**: MOI_x, determined at break-section with respect to the neutral axis, was not significantly different between the two groups (*t*-test $P = 0.075$). **D**: Maximum force was measured and did not differ between the control and the fluoxetine-treated groups. **E**: Strain was calculated and also did not differ between the control and fluoxetine-treated animals. **F**: Stress was significantly lower in the fluoxetine-treated animals compared to control animals (*t*-test $P = 0.006$). **G**: Young's modulus was significantly lower in the fluoxetine-treated group (Mann–Whitney *U* test $P = 0.029$). Results are given as means + 95%-CI, $n_{control} = 10$ and $n_{fluox} = 11$.

architecture or bone mechanical properties in female rats. We did, however, find that trabecular bone thickness in the metaphysis of the femur was significantly lower in the fluoxetine-treated animals, suggesting decreased bone formation and/or increased bone resorption. Endocortical bone volume was larger in fluoxetine-treated animals, suggesting increased endocortical bone resorption. These results are in agreement with our in vitro results, where we found that osteoblastic proliferation was inhibited by fluoxetine administration, and OPG/RANKL ratio was decreased, suggesting a stimulation of osteoclastic differentiation [Gustafsson et al., 2006b]. The absence of osteoclasts at the end point of the study (bone histomorphometry), does not exclude a difference in resorption between controls and treated animals anytime during the fluoxetine treatment. Total bone strength of the diaphysis was not affected by fluoxetine treatment, however, bone tissue strength was significantly

lower in the fluoxetine-treated animals. A compensatory mechanism for the decreased bone quality may be acting upon the bone architecture leading to a higher cross-sectional MOI_x in the fluoxetine-treated animals, leading to equal bone strength.

The exact mechanisms of serotonin action on bone metabolism are still unclear. Serotonin may act directly on the receptors on the bone cells, or may also act indirectly via other factors important in bone metabolism, like leptin and adiponectin [Ducy et al., 2000; Takeda et al., 2002; Gordeladze and Reseland, 2003; Luo et al., 2005; Oshima et al., 2005]. It is known that systemic injection of serotonin and its precursor, 5-hydroxytryptophan, elevates leptin levels in mice [Yamada et al., 1999, 2000]. Adiponectin levels have been shown to increase after administration of a $5\text{-}HT_{2A}$ receptor antagonist, indicating that the serotonergic system may influence adiponectin levels [Yamakawa et al., 2003]. Furthermore, some studies suggest an effect of anti-depressants on body weight and energy expenditure [Dryden et al., 1996; Fava et al., 2000; Moosa et al., 2003]. We found no significant change in body weight in the fluoxetine-treated animals, however, we did find that fluoxetine-treated animals had a non-significant higher fat percentage at every time point. To investigate a possible role for leptin and adiponectin in this study, we determined circulating leptin and adiponectin levels in these animals. We, however, found no significant difference in leptin and adiponectin plasma levels between the fluoxetine-treated animals and controls, indicating that these factors were not involved in the differences in bone metabolism found in this study.

The fact that we only see slight differences between control animals and fluoxetine-treated animals may lie in the dose we have used (5 mg/kg/day), however, this dose is 0.6 time the maximum recommended human dose (MRHD), serum levels are at similar levels as in patients, and other studies, showing significant fluoxetine effects, have used this dose in rats [Wong et al., 1988; Hsiao et al., 2006]. The fact that others have used this dose with success indicates that the serotonin transporter is sensitive for fluoxetine when this dose is used. However, we cannot be sure that the serotonin transporter in bone is equally affected as transporters in other tissues. Of course the best way to study the effects of fluoxetine on bone metabolism seems to be cohort studies on the large number of patients that use fluoxetine. Many studies have focused on the correlation between depression on one side and BMD, fall incidence, and fracture risk on the other. There are, however, some problems in the comparison of published data, for instance differences in gender, age, depression-surveys, medication, physical exercise, and dietary calcium-intake, but also whether these factors were taken into account during the analysis of the data, make comparison of the data virtually impossible. In the current study we solely investigated the effects of fluoxetine on bone metabolism. In summary, we find indications for higher bone resorption and slightly diminished bone quality in the fluoxetine-treated rats, which seems to be compensated by an adaptation of the bone geometry. Overall, bone strength was not different in fluoxetine-treated animals compared to control animals.

Bolo et al. [2004] reported on accumulation of fluoxetine and/or fluoxetine metabolites, in the bone marrow after long-term treatment with fluoxetine. Up to months after the treatment was stopped, plasma and brain levels were undetectable, but fluoxetine and nor-fluoxetine was still detectable in the bone marrow. Accumulation of fluoxetine and nor-fluoxetine in bone marrow may result in long-term interference of the serotonergic system in bone tissue, bone cells, and their precursors, even after treatment has stopped. Despite the minor effects we find in this study we feel that caution should be taken until further clinical data exist, since the effects of long-term treatment with SSRIs, such as fluoxetine, on bone tissue in patients have not yet been studied and we do not know the effects on the long-run after treatment.

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