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Linking Chronic Tryptophan Deficiency With Impaired Bone Metabolism and Reduced Bone Accrual in Growing Rats

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ABSTRACT

There is increasing evidence that serotonin may regulate bone metabolism. However, its role remains to be clarified. Serotonin seems to be either beneficial or detrimental for bone tissues depending on the pharmacological manipulation used. In this study we evaluated the impact of a reduction of serotonergic stores induced by chronic tryptophan (TRP) depletion on various bone parameters in growing rats. For this purpose rats received a TRP-free diet for 60 days. Bone mass, mineral content and density were measured by DXA and by pQCT in the appendicular skeleton. Bone metabolic markers included urinary deoxypyridinoline and serum osteocalcin measurements. IGF-I levels were also evaluated. In TRP-free diet rats, we found a decrease in body weight, a delayed femoral bone growth and bone mineral content as measured by DXA. pQCT analysis showed that these effects were related to a reduction of both cortical and trabecular bone and are associated with a reduction of bone strength. These effects are due to a negative shift in the balance between bone formation and resorption with a significant decrease in bone formation as evidenced by a reduction both in osteocalcin and IGF-I levels. The present data extend our overall knowledge on the participation of serotonin in the regulation of growing bone and could be of interest in studying the impairment of bone growth in depressed subjects under particular condition of rapid bone accrual such as childhood and adolescence. J. Cell. Biochem. 107: 890–898, 2009.

KEY WORDS: BONE METABOLISM; BONE MASS; TRYPTOPHAN; SEROTONIN DEPLETION

Serotonin (5-HT) regulates many behavioral systems in the CNS and has a role in cardiovascular, gastrointestinal and circulatory physiology. In recent years, in addition to these well-established activities of 5-HT, much attention has focused on the possible role of 5-HT in the regulation of bone turnover [Rosen, 2009]. Serotonin is synthesized from the naturally occurring essential amino acid thryptophan by a two-step pathway in which tryptophan hydroxylase is the rate-limiting enzyme. The activity of 5-HT is regulated by the 5-HT membrane transporter (5-HTT) which is highly specific for the internalization of 5-HT. Thus, it is considered a key protein in 5-HT signaling and metabolism. The wide-spread actions of 5-HT results from its binding to multiple receptors [5-HTRs, Jonnakuty and Gragnoli, 2008].

Osteoblasts, osteocytes, and osteoclasts express mRNA for tryptophan-hydroxylase, [Bliziotes et al., 2001, 2006; Gustafsson et al., 2006a] and for 5-HTT. Furthermore, functional 5-HTRs have been detected in osteoblasts [Collet et al., 2008], suggesting that bone cells present a functional system for both responding to and regulating 5-HT activity. The role of 5-HT in the control of bone remodeling remains to be clarified. In vitro studies indicate an osteogenic and/or antiresorptive effect of 5-HT. Serotonin, in fact, stimulates OB recruitment and proliferation [Collet et al., 2008], increases osteoprotegerin and decreases receptor activator of NF-κB ligand (RANKL) secretion from osteoblasts thus reducing osteoblast signaling for osteoclast differentiation and activation [Gustafsson et al., 2006a].

However, in vivo studies do not strongly indicate whether 5-HT is beneficial or detrimental to bone tissues since contrasting results have been reported. Some evidence suggests that 5-HT administration and the inhibition of the 5-HTT are beneficial to the skeleton. Long-term 5-HT administration in growing rats leads to high bone mineral density, an increase in endosteal bone apposition and bone stiffness of long bones and reduced bone resorption [Gustafsson et al., 2006b]. Furthermore, Collet et al. [2008] found that 5-HT_{2B}R knockout mice displayed reduced bone density due to reduced bone formation. The potential beneficial effect of 5-HT on bone has been supported by the indirect evidence obtained with 5-HTT inhibition. It has been reported that 5-HTT inhibition had an

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anabolic effect on trabecular bone [Battaglino et al., 2007] and that 5-HTT inhibition ameliorates the negative skeletal effects of chronic mild stress [Yirmiya et al., 2006]. By contrast to these beneficial activities of 5-HT on bone, are the data obtained in mice genetically lacking the 5-HTT. Knockout mice for 5-HTT present reduced bone mass, altered bone architecture and inferior mechanical properties compared to their control littermates [Warden et al., 2005, 2008]. The same results were obtained in mice [Warden et al., 2005, 2008] and rats [Bonnet et al., 2007; Westbroek et al., 2007] treated with the selective 5-HT reuptake inhibitor (SSRI), fluoxetine. The preclinical studies demonstrating a negative effect of 5-HTT inhibition on bone are in agreement with evidence provided by clinical studies showing that the use of SSRIs increased rate of bone loss both in elderly women [Diem et al., 2007] and men [Haney et al., 2007].

These conflicting data on the role of 5-HT in the control of bone mass, could be due to different experimental procedures and/or to the pharmacological manipulations used to modulate 5-HT neurotransmission. A non-pharmacological approach to investigate the effects of reduced 5-HT tone in the control of bone mass and bone remodeling, could be represented by chronic tryptophan (TRP) depletion, an experimental tool previously used to clarify the role of 5-HT in behavioral and psychiatric illness [Bell et al., 2001; Cahir et al., 2007]. In fact, the conversion of TRP to 5-hydroxy-TRP is the first step in the 5-HT synthesis. Since body cannot synthesize TRP, chronic TRP depletion can be achieved by reducing dietary sources of TRP.

It is well known that depressive disorders involve the 5-HT system and that low bone mass is frequent in depressed subjects [Cizza et al., 2001]. Most of these data derived from studies performed in adult or elderly, whereas little is known about the impact of depression on bone accrual in the growing skeleton. This issue deserves to be addressed since 10% of children and adolescents suffer from depression [Birmaher et al., 1996] and the acquisition of bone mass during adolescence is the most important determinant of the achievement of peak bone mass [Harel, 2008].

Therefore, the aim of the present study was to examine the effects of a TRP-deficient diet on the bone mineral accrual in the growing rat skeleton. The effects of the TRP-deficient diet on bone were examined by measuring Bone Mineral Content (BMC) and density (BMD) by DXA and by pQCT in the appendicular skeleton. The biochemical markers of osteoblast and osteoclast activities were also evaluated. The results of the present study could add new inside in the knowledge of the neuronal communication networks in bone.

MATERIALS AND METHODS

ANIMALS

Male Sprague-Dawley rats 250–275 g were purchased from Charles River Laboratories (Calco, Varese, Italy). All rats were housed in single cages under controlled conditions ($22 \pm 2^{\circ}C$, 65% humidity, 12 h light/12 h darkness cycle). All experiments were approved by the Institutional Animal Care Committee and animals were maintained in accordance with the European Union Guidelines for the care and use of laboratory animals.

EXPERIMENTAL PROTOCOL

Rats were allowed to acclimatize for 2 weeks and then were randomly divided into three groups of eight rats each: one group of controls (A) received a standard diet containing 0.25% TRP with food ad libitum; one group (B) received a standard diet and were pair-fed with food matched to that consumed by the group C on a daily basis; one group (C) received a TRP-free diet with food ad libitum. The amount of food administered to pair-fed rats was established by weighing the food consumed by rats fed with test diets for 60 days. Food intake and body weight were assessed every day and every week, respectively. Animals had free access to water at all times.

TRP-free and standard control diet were purchased from Piccioni s.r.l. (Gessate, Milano, Italy). The TRP-free diet had the following composition: sucrose plus mais starch 65.1% and vitamin mix integration 1%, gelatine 11.3%, amino acids (L-glycine, L-lysine, L-histidine, L-methionine, L-phenylalanine, L-leucine, L-isoleucine, L-threonine, L-valine, glutamic, and aspartic acids) 7.6%, cellulose 2%, mais oil 8%, ammonium tartrate 1.3%, AIN-76 salt mix 3.5%, choline HCl 0.2%. Standard diet had identical composition except that 0.25% of TRP was added in place of an equivalent amount of sucrose.

Bone areas, bone mineral content (BMC) and bone mineral density (BMD) were measured at baseline (t_0), 30 (t_{30}), and 60 (t_{60}), days by DXA. Twenty-four hour urine samples were collected from rats housed in metabolic cages (Techniplast, Varese, Italy) at t_0, t_{30}, and t_{60} for the measurement of deoxypyridinoline (DPD), a marker of bone resorption. Samples were immediately frozen and stored at $-20^{\circ}C$ until assayed. Urine samples were also tested for 24 h excretion of 5-hydroxyindolacetic acid (5-HIAA), the metabolite of 5-HT. Blood samples were drawn at t_0, t_{30}, and t_{60} under light ether anesthesia, by cardiac puncture for the measurement of osteocalcin (OC), a marker of bone formation, and for IGF-I measurement. Plasma was stored at $-80^{\circ}C$ until assayed.

At the end of the experiment, the animals were euthanized and femora and tibiae were excised and collected for quantitative computed tomography (pQCT) examination.

DUAL ENERGY X-RAY ABSORPTIOMETRY (DXA)

All rats were anaesthetized with Zoletil, 40 mg/kg, i.m. (Virbac, Italy) and scanned with a Hologic QDR-4000 instrument, (Hologic Inc., Waltham, MA) in the ultra-high resolution mode. Longitudinal line spacing of 0.254 mm was used, implemented with 1.0 mm diameter collimator and with High Resolution Software (version 4.47) adapted for small animals. Three regions of interest were chosen: the entire femur, the femoral metaphysis and diaphysis. The software provided the total area (cm^2) of the planar image of the selected segments, the BMC, in mg, and the BMD in mg/cm^2. Coefficients of variation were 3% for BMC and 1% for BMD. The precision and accuracy of DXA in small laboratory animals have been widely validated [Gala Paniagua et al., 1998].

pQCT MEASUREMENTS

pQCT measurements were performed using a Stratec Research SA + pQCT scanner (Stratec Medizintechnik GmbH, Pforzheim, Germany) with a voxel size of 100 μm and a scan speed of 7 mm/s.

The excised bones were fixed with manufacturer-made plastic holder for the pQCT measurements. The correct longitudinal positioning was determined by means of an initial "scout scan." Femora and tibiae were examined at the distal metaphysis (three sections measured between 3 and 5 mm of the end of the bone) and at the midshaft (two sections at 15 mm from knee joint space). The scans were analyzed with pQCT software 6.00B using contour mode 1 and peel mode 2 with a threshold of 400 mg/cm^3 for the calculation of trabecular and total bone parameters at the metaphysis and with a threshold of 710 mg/cm^3 for cortical bone parameters at the diaphysis. In each transverse image, area, BMC, BMD, endosteal, and periosteal perimeter were measured. The polar bone strength strain index (SSI, m^{-3}) was measured at the femur and tibia diaphysis and calculated by the manufacturer's software as follows: $SSI = \Sigma_i =_{1,n} r_i^2 \cdot aCD/ND \cdot r_{max}$, where r is the distance of a voxel from the center of gravity, r_{max} is the maximum distance of a voxel from center of gravity, a is the area of a voxel, CD is the cortical density and ND is the normal physiological density equal to 1,200 mg/cm^3.

BIOCHEMICAL ANALYSIS

Total urinary DPD levels were extracted using a commercial kit (ChromSystems GmbH, Munchen, Germany) and then measured by fluorimetric detector HPLC. Intra- and inter-assay variations were 5.5% and 3.1%, respectively. The total daily excretion DPD was corrected for creatinine excretion. Urinary creatinine was measured colorimetrically using a commercial kit (Quidel Corporation, San Diego, CA). Total serum OC was determined using a commercial immunoradiometric assay kit (Immunotopics, CA). The intra-assay variation was 4%, while the inter-assay variation was 7%. Daily urinary excretion of 5HIAA was measured using a commercial immunoassay kit (DRG International Ink., USA). The intra-assay variation was 11%, while the inter-assay variation was 10.3%. Serum IGF-I was measured using a commercial EIA kit (IDS, UK). The intra-assay variation was 6.8%, while the inter-assay variation was 7.3%.

STATISTICAL ANALYSIS

Statistical analysis was performed using a statistical package (PRISM, vers. 2.01 GraphPad Software San Diego, CA). Data are reported as the means ± SEM. Densitometric and biochemical results were analyzed by one-way repeated measures analysis of variance (ANOVA) followed by Dunnett's test. Differences between groups were analyzed by Bonferroni test. Since the animals were in the growing phase, the results of DXA were analyzed as % changes relative to baseline values.

RESULTS

EFFECT OF CHRONIC TRP DEPLETION ON RAT BODY WEIGHT AND FOOD INTAKE

As shown in Figure 1, compared to baseline values, control group weight significantly increased throughout the experimental period.

Accordingly to the previous reports of D'Souza et al. [2004], TRP-free diet rats had a sustained and significant reduction in body weights compared with those detected in the control group. Pair-fed rats showed a significant lower growth curve than the control group. This reduction was less marked than the one observed in TRP-free diet animals. The reduction of body weight was associated with a lower food consumption (data not shown).

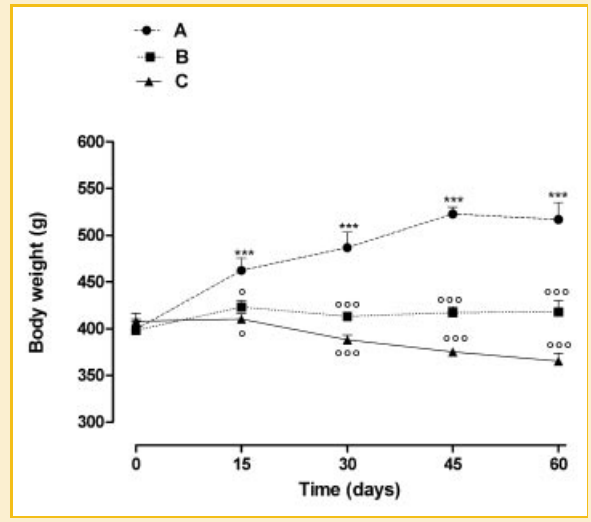

Fig. 1. Effects of the tryptophan (TRP)-free diet on body weight. Data represent means ± SEM of 8 rats/group receiving control standard diet (A), standard diet pair-fed (B) and TRP-free diet (C). $^{***}P < 0.001$ versus t_0 values; $^{\circ}P < 0.05$, $^{\circ\circ\circ}P < 0.001$ versus A.

The TRP-free diet produced a significant reduction in urinary excretion of 5-HIAA compared with rats given the control standard diet, whereas no significant difference was observed in pair-fed rats (Fig. 2).

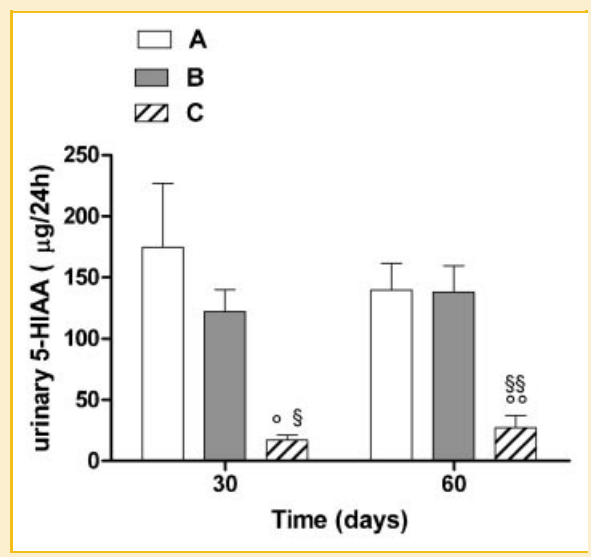

Fig. 2. Effects of the tryptophan (TRP)-free diet on the urinary levels of 5-hydroxyindole acetic acid (5-HIAA). Data represent means ± SEM of 8 rats/group receiving control standard diet (A), standard diet pair-fed (B) and TRP-free diet (C). $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ versus A; $^{\$}P < 0.05$; $^{\$\$}P < 0.01$ versus B.

BIOCHEMICAL PARAMETERS OF BONE TURNOVER AND SERUM IGF-I

In control rats, the urinary excretion of DPD significantly decreased with time. This reflects changes in bone turnover which is known to be higher in young rats as compared with adults. Similar results were obtained in pair-fed rats. In TRP-free diet treated rats there was a more slight reduction in the urinary excretion of DPD than in the other experimental groups as evidenced by the lack of a significant reduction of DPD excretion as compared with baseline values (Fig. 3a). Consistent with the coupling of bone resorption to bone formation, OC levels decreased with time similarly to those of DPD in control and pair-fed rats. In TRP-free diet treated rats there was a more marked decrease in serum OC which reaches statistical significance compared with both control and pair-fed rats at t_{60} (Fig. 3b).

All rats had circulating IGF-I levels significantly lower at t_{60} as compared with values detected at baseline. In pair-fed rats the reduction of IGF-I levels reaches −26% as compared to controls. In TRP-free diet treated rats, was observed a more sustained reduction in serum IGF-I (−67%) which was statistically different as compared with both control and pair-fed rats (Fig. 4).

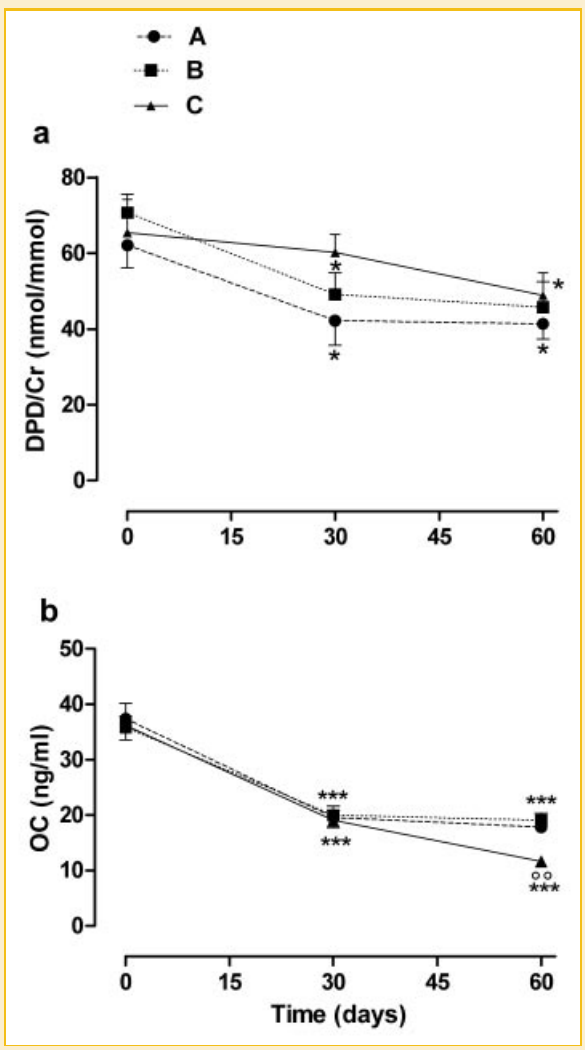

Fig. 3. Effects of the tryptophan (TRP)-free diet (a) on the urinary excretion of dexoypyridinoline (DPD) and (b) on serum osteocalcin (OC) levels. Data represent means ± SEM of 8 rats/group receiving control standard diet (A), standard diet pair-fed (B) and TRP-free diet (C). $^{*}P < 0.05$, $^{***}P < 0.001$ versus t_0 values; $^{\circ\circ}P < 0.001$ versus A.

DXA OF THE FEMUR

Total area, BMC and BMD of the femur significantly increased compared with the baseline in all the experimental groups. The effect of daily assumption of TRP-free diet was a significant reduction of total femoral area, BMC and BMD values compared with those measured in controls throughout the experimental period. In pair-fed rats the increment with age of femoral BMC and BMD was lower than in controls. This trend, however, did not reach statistical significance as compared with controls (Fig. 5).

The reduction of both BMC and BMD in TRP-free diet rats were observed in a region of the femur that contains both cortical and trabecular bone (femoral metaphysis) as well as in femoral diaphysis, a region that contains only cortical bone (Figs. 6 and 7).

In pair-fed rats a reduction of both BMC and BMD was detected only at femoral metaphysis, at the end of the experiment. This trend, however, did not reach statistical significance as compared with controls (Fig. 6).

pQCT OF THE FEMUR AND TIBIA

As shown in Tables I and II, total bone area of both distal and middle femur significantly decreased in TRP-free diet rats compared with controls as a result of a reduced cortical and cortical/subcortical bone area, probably due to a decrease of radial growth as evidenced by a reduction of both periosteal and endosteal perimeters. Volumetric trabecular density significantly decreased in TRP-free rats compared with controls; the simultaneous decrease in bone area and BMC detected in cortical/subcortical bone of femoral

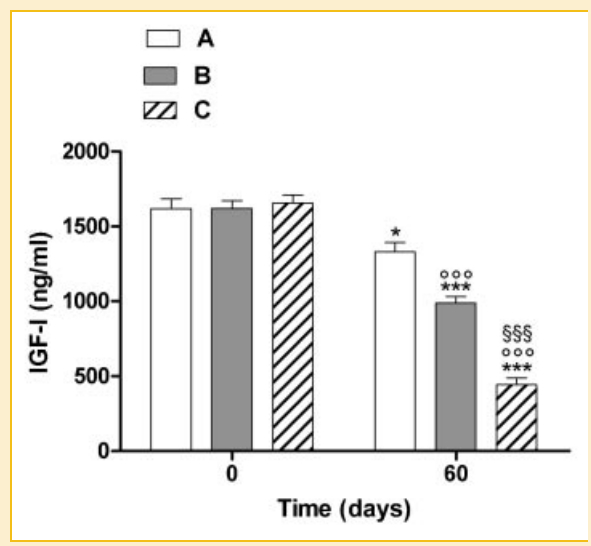

Fig. 4. Effects of the tryptophan (TRP)-free diet on serum IGF-I levels. Data represent means ± SEM of 8 rats/group receiving control standard diet (A), standard diet pair-fed (B) and TRP-free diet (C). $^{*}P < 0.05$; $^{***}P < 0.001$ versus t_0 values; $^{\circ\circ\circ}P < 0.001$ versus A; $^{\S\S\S}P < 0.001$ versus B.

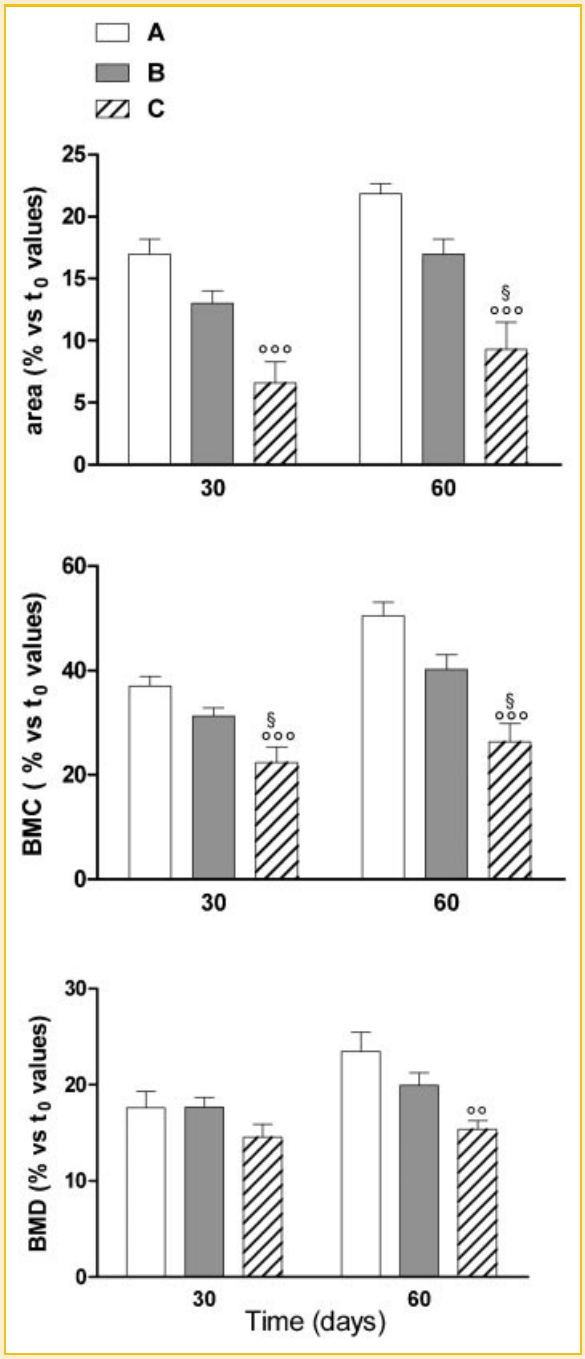

Fig. 5. Effects of the tryptophan (TRP)-free diet on total femoral area, BMC and BMD. Data are expressed as percent changes from t_0 values. Data represent means ± SEM of 8 rats/group receiving control standard diet (A), standard diet pair-fed (B) and TRP-free diet (C). °° $P < 0.01$; °°° $P < 0.001$ versus A; § $P < 0.05$ versus B.

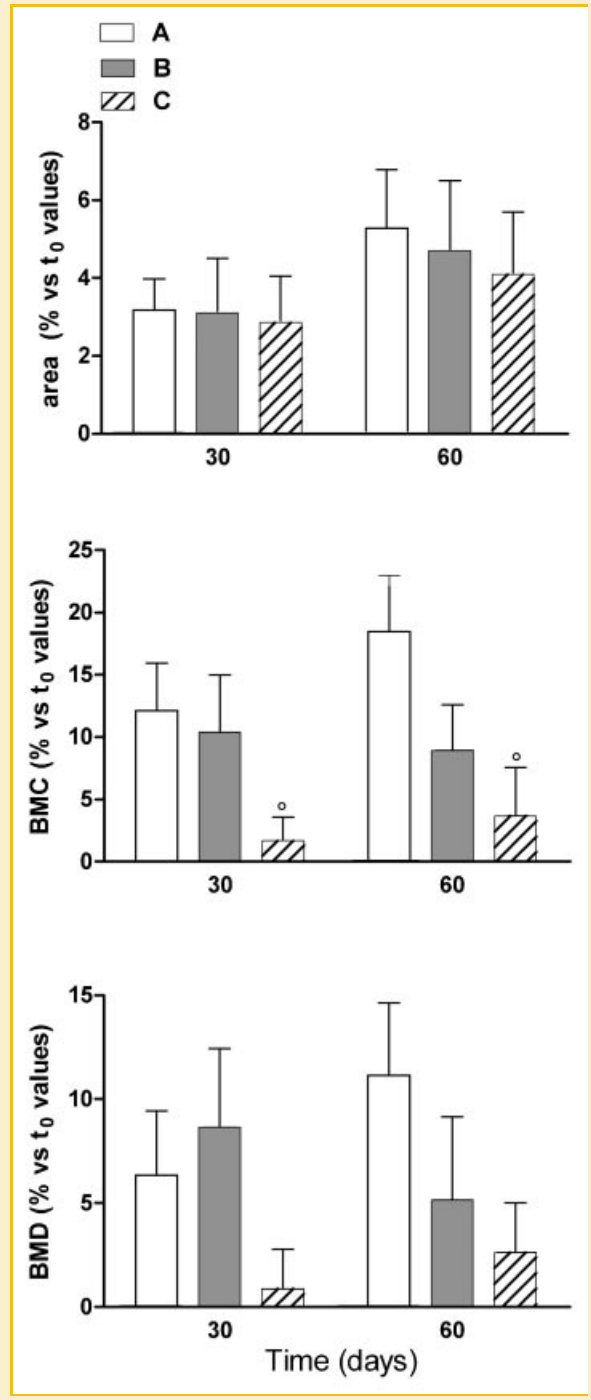

Fig. 6. Effects of the tryptophan (TRP) - free diet on bone area, BMC and BMD measured at femoral metaphysis. Data are expressed as percent changes from t_0 values. Data represent means ± SEM of 8 rats/group receiving control standard diet (A), standard diet pair-fed (B) and TRP-free diet (C). ° $P < 0.05$ versus A.

metaphysis (Table I) and in cortical bone of femoral diaphysis (Table II) could account for the unchanged BMD values. TRP deficiency resulted in decreased bone strength as shown by the SSI parameter detected at femoral diaphysis (Table II).

In pair-fed rats was observed a significant decrease of total bone BMC at femoral metaphysis which seems to depend on a reduction in cortical/subcortical bone (Table I). A trend throughout a reduction in cortical bone, was observed at femoral diaphysis. However, it did not reach statistical significance compared with controls (Table II). At variance from TRP-free diet rats, in pair-fed rats no significant difference was observed in trabecular bone (Table I). Similar results were obtained in tibial metaphysis and diaphysis (Tables I and II).

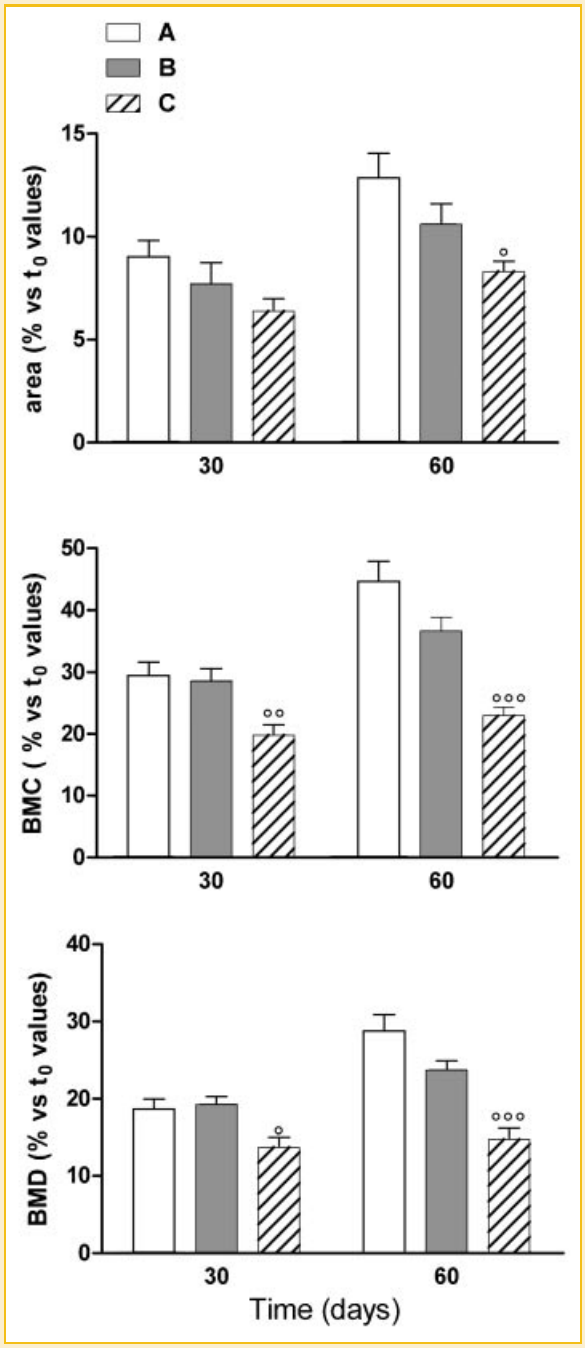

Fig. 7. Effects of the tryptophan (TRP)-free diet on bone area, BMC and BMD measured at femoral diaphysis. Data are expressed as percent changes from t_0 values. Data represent means ± SEM of 8 rats/group receiving control standard diet (A), standard diet pair-fed (B) and TRP-free diet (C). $^{\circ}P < 0.05$; $^{\circ\circ}P < 0.01$; $^{\circ\circ\circ}P < 0.001$ versus A.

DISCUSSION

Our study indicates that TRP-deficiency is associated with growth retardation, and impairment of the normal process of bone mass acquisition of the appendicular skeleton. In fact, we found a decrease in body weight in growing rats receiving TRP-free diet which was accompanied by a delayed bone growth (as shown by bone areas) and mineral accrual (BMC) at both femur and tibia. The reduction of BMC was more evident at the sites containing both cortical and trabecular bone as the distal femur, as shown by DXA analysis. Accordingly, pQCT measurements performed at femoral and tibia metaphysis showed a reduction of both trabecular and cortical/subcortical bone. The impairment of cortical bone following TRP deficiency was confirmed by the pQCT analysis performed at femoral and tibial diaphysis. In fact, at this skeletal site, was detected a decreased cortical bone size due to a decreased radial growth which involves a reduction of either periosteal and endosteal bone formation. Furthermore, TRP-free diet administration seems to reduce bone elasticity. This was especially pronounced with regard to the femoral and tibia diaphysis, where TRP-deficiency led to a decrease in biomechanical parameters as shown by a reduction of SSI values. Our data are in agreement with a beneficial skeletal effect of 5-HT and fit well with the previous result of Gustafsson et al. [2006b] showing that long-term 5-HT administration positively affects bone architecture and leads to higher bone stiffness in the rat.

It is well known that growing rats (from 2 to 5 months old) increased bone mass by longitudinal bone growth and periosteal expansion [Ortoft et al., 1999]. It has also been reported that, starting from 2 months onwards, the cancellous bone mineralizing surface decrease, denoting an age-related reduction in bone turnover markers [Ortoft et al., 1999].

In the present study, there was a significant decrease in markers of bone resorption (DPD) and bone formation (OC) in control rats during growth. However, in TRP-free diet rats we found a negative shift in the balance between bone formation and bone resorption, as shown by the biochemical data, which could account for the negative effects of TRP-deficiency on bone mass.

The effects of TRP-free diet on bone mass and metabolism seems to be related to a reduction of 5-HT synthesis as evidenced by the marked decrease in urinary 5-HIAA detected in this experimental group.

Recent findings have shown the presence of functional 5-HT pathways in bone [Bliziotes et al., 2001; Battaglino et al., 2004]. The majority of in vitro studies indicate that 5-HT may have beneficial effects on the skeleton. Serotonin, in fact, stimulates osteoblast and osteocyte proliferation and reduced osteoblastic signalling for osteoclastic differentiation and activation by increasing osteoprotegerin and decreasing RANKL secretion from osteoblasts [Bliziotes et al., 2001, 2006; Gustafsson et al., 2006a]. However, contradictory data on the role of 5-HT on bone mass have been reported in preclinical studies. Our data indicate that the adverse effects of TRP deficiency on bone mass could be the consequence of its ability to impair bone cell activities. Initially (t_{30}), TRP-deficiency results in a phase characterized by bone resorption exceeding bone formation that is then followed by a phase of reduced bone formation (t_{60}). The evidence that no significant changes in DPD excretion were detected between TRP-free diet rats and controls, whereas TRP deficiency induced a significant greater decrement of OC levels, indicate that an availability reduction of 5-HT is mainly associated with a decrease in bone formation. This point, however, deserves further histomorphometric evaluation. Our results are in agreement with previous studies suggesting a beneficial bone effect of long-term 5-HT administration [Gustafsson et al., 2006b]. Other

TABLE I. pQCT Measurements of Femoral and Tibial Metaphysis in Rats Receiving Control Standard Diet (A), Standard Diet Pair-Fed (B), and TRP-Free Diet (C)

	Femur			Tibia		
	A	B	C	A	B	C
Total bone						
Area (mm^2)	25.50 ± 0.76	22.82 ± 1.06$^{\circ\circ}$	22.08 ± 0.69$^{\circ\circ\circ}$	32.83 ± 1.04	28.14 ± 1.10$^{\circ\circ}$	25.06 ± 1.45$^{\circ\circ}$
BMC (mg/mm)	14.28 ± 0.30	12.15 ± 0.40$^{\circ\circ}$	11.48 ± 0.30$^{\circ\circ\circ}$	16.51 ± 0.44	13.77 ± 0.48$^{\circ\circ}$	12.24 ± 0.54$^{\circ\circ\circ}$
BMD (mg/cm^3)	563.10 ± 21.2	536.60 ± 14.6	522.20 ± 8.30	509.50 ± 14.6	499.70 ± 9.3	496.20 ± 10.2
Trabec bone						
Area (mm^2)	11.27 ± 1.23	11.38 ± 0.74	11.32 ± 0.40	15.21 ± 1.87	14.00 ± 0.72	12.68 ± 0.83
BMC (mg/mm)	2.26 ± 0.20	2.63 ± 0.10	1.74 ± 0.08°	3.49 ± 0.24	2.84 ± 0.17	2.03 ± 0.14$^{\circ\circ\circ\S}$
BMD (mg/cm^3)	205.50 ± 13.6	178.90 ± 8.4	152.20 ± 8.4$^{\circ\circ}$	227.90 ± 11.9	196.60 ± 8.0	145.50 ± 7.1$^{\circ\circ\circ\S}$
Cort/subc bone						
Area (mm^2)	14.23 ± 0.81	11.44 ± 0.60°	10.77 ± 0.40$^{\circ\circ}$	17.62 ± 0.78	14.15 ± 0.68°	12.44 ± 0.65$^{\circ\circ\circ}$
BMC (mg/mm)	12.05 ± 0.50	10.09 ± 0.40°	9.73 ± 0.20$^{\circ\circ}$	13.03 ± 0.45	10.93 ± 0.37$^{\circ\circ}$	10.21 ± 0.41$^{\circ\circ\circ}$
BMD (mg/cm^3)	863.50 ± 26.5	901.60 ± 21.2	922.40 ± 17.5	752.90 ± 15.8	798.70 ± 13.3	849.90 ± 12.3$^{\circ\circ\circ\S}$
Periosteal per (mm)	17.82 ± 0.27	16.68 ± 0.33	16.11 ± 0.29°	19.57 ± 0.34	17.72 ± 0.42°	17.28 ± 0.40$^{\circ\circ}$
Endosteal per (mm)	14.83 ± 0.40	13.84 ± 0.30	13.37 ± 0.29°	16.57 ± 0.43	14.83 ± 0.45°	14.46 ± 0.39$^{\circ\circ}$

$^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$, $^{\circ\circ\circ}P < 0.001$ versus A; $^{\S}P < 0.05$ versus B.

evidence for a possible beneficial effect of 5-HT on the skeleton was provided by Yirmiya et al. [2006]. They found that a depressive state in mice, characterized by dysfunction of 5-HT neurotransmission, resulted in negative skeletal effects due to a decrease in bone formation rate.

On the contrary, a potential detrimental role for 5-HT for skeletal health has been suggested by data obtained in mice with a null mutation in the gene encoding for the 5-HTT and in rodents treated with SSRI, fluoxetine [Warden et al., 2005, 2008]. These discrepancies could be due to the different experimental procedures or animal species (mice/rats) used or to the fact that the marked increase in extracellular 5-HT concentration after fluoxetine, could have amplified the duration of 5-HT signalling at 5-HT receptors located on bone cells. It is worth noting that, in contrast to the extracellular fluid levels, 5-HT brain tissue concentration is decreased in serotonin transporter gene knockout mice [Murphy and Lesch, 2008]. Yet, it is unclear whether the negative skeletal effect of SSRI depends on changes in extracellular and intracellular 5-HT levels or to 5-HT receptor activation and adaptation as previously reported in brain tissues.

The possible different role of 5-HT cytoplasmatic and extracellular levels on bone cell activities has been suggested by Battaglino et al. [2004]. They found that fluoxetine inhibits osteoclast differentiation from mouse bone marrow cells, whereas exposing these cells to reserpine, which inhibits the transport of 5-HT into cytoplasmatic vesicles, increases osteoclast differentiation. Therefore, it is unlikely that elevation in cytoplasmatic rather than extracellular levels of 5-HT is necessary for the regulation of bone cell activity. Another possible explanation for the results obtained with fluoxetine on bone mass comes from previous studies showing that the compound is able not only to inhibit 5-HTT, but also to directly interact with 5-HT2 receptors [Pälvimäki et al., 1996; Ni and Miledi, 1997; Koch et al., 2002; Gustafsson et al., 2006a]. Thus it is possible that the negative effect of fluoxetine on bone could be mediated, at least in part, through an interaction with these receptors.

Our results on the negative bone effects of TRP-free diet are in apparent contrast with those recently reported by Yadav et al. [2008]. In this study they found a lack of effect of a 75% TRP-less diet on vertebral bone mass in mice. The reason for this discrepancy

TABLE II. pQCT Measurements of Femoral and Tibial Diaphysis in Rats Receiving Control Standard Diet (A), Standard Diet Pair-Fed (B) and TRP-Free Diet (C)

	Femur			Tibia		
	A	B	C	A	B	C
Total bone						
Area (mm^2)	14.89 ± 0.51	13.69 ± 0.44	12.95 ± 0.17$^{\circ\circ}$	10.00 ± 0.40	9.18 ± 0.30	8.76 ± 0.24°
BMC (mg/mm)	12.23 ± 0.24	11.06 ± 0.30	10.75 ± 0.30$^{\circ\circ}$	8.65 ± 0.26	7.99 ± 0.21	7.74 ± 0.12°
BMD (mg/cm^3)	824.40 ± 17.2	809.2 ± 15.8	829.20 ± 17.1	867.4 ± 15.3	871.9 ± 11.4	886.7 ± 17.1
Cort bone						
Area (mm^2)	7.97 ± 0.17	7.20 ± 0.20°	7.00 ± 0.18$^{\circ\circ}$	5.74 ± 0.16	5.29 ± 0.14	5.16 ± 0.07°
BMC (mg/mm)	10.87 ± 0.50	9.78 ± 0.28	9.57 ± 0.24°	7.59 ± 0.22	7.05 ± 0.19	6.87 ± 0.12°
BMD (mg/cm3)	1363 ± 5.4	1360 ± 6.1	1367 ± 6.1	1323 ± 3.7	1324 ± 5.4	1331 ± 6.7
Periosteal per (mm)	12.97 ± 0.16	12.36 ± 0.21	12.02 ± 0.14$^{\circ\circ}$	11.73 ± 0.41	10.94 ± 0.38	10.54 ± 0.23°
Endosteal per (mm)	8.28 ± 0.31	7.93 ± 0.21	7.32 ± 0.12°	5.96 ± 0.24	5.48 ± 0.22	5.40 ± 0.17
SSI	11.65 ± 0.68	10.19 ± 0.47	9.49 ± 0.23$^{\circ\circ}$	6.10 ± 0.27	5.46 ± 0.80	5.20 ± 0.15°

$^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ versus A.

could depend on the different skeletal sites examined (axial/appendicular skeleton). Interestingly, we found that TRP-deficiency did not significantly modify DXA bone parameters in the lumbar vertebrae of growing rats (data not shown). This suggests that, in condition of TRP-deficiency, there is a region specific alteration in the bone mineral accrual which could be detected only in the appendicular skeleton.

In agreement with previous studies [D'Souza et al., 2004], we found that TRP deficiency reduced food intake and body weight. Body growth regulates skeletal growth as a result of increased mechanical loading on the skeleton that is perceived by bone cells as osteogenic [Bikle et al., 2003; Dufour et al., 2007]. Weight-bearing and mechanical stresses are important determinants of cortical bone mass. In particular, increased loading of long bones is associated with a greater mechanical stress on the subperiosteal surface and increases bone formation by subperiosteal expansion [Zhang and Yokota, 2007].

Therefore, a reduction of body weight, could have contributed to the negative skeletal effects detected on cortical bone in TRP-free diet rats. This bias has been controlled by using TRP-normal diet treated rats which were pair-fed relative to the TRP-free diet animals. DEXA analysis of bone parameters detected in the femoral metaphysis of TRP-normal diet pair-fed rats shows only a trend throughout a reduction in all bone parameters which did not reach statistical significance compared with controls. These data together with the results obtained by pQCT analysis performed in both femoral and tibial metaphysis showing only a reduction of cortical/subcortical area and BMC in pair-fed rats, indicate that the negative skeletal effects detected in TRP-free diet rats could be minimally influenced by a reduction of weight-bearing and mechanical stresses. Our data showing that in TRP-free diet rats there was a more marked decrease of cortical bone compared with the one detected in pair-fed animals, suggests that the lack of 5-HT might have influenced mechano-transduction. So, it is possible that 5-HT deficiency could have reduced skeletal responsiveness to mechanical loading thus reducing bone mineral accrual. In line with this assumption are in vitro studies showing that the amine modulate the response of osteoblasts to mechanical stimulation [Westbroek et al., 2001].

Experimental and clinical studies indicate that dietary protein restriction, by influencing both production and function of growth factors, particularly IGF-I, could control bone anabolism [Rosen, 1994; Rosen and Donahue, 1995; Canalis and Agnusdei, 1996]. Protein restriction has been shown to reduce IGF-I plasma levels by inducing a resistance to the action of GH at the hepatic level [VandeHaar et al., 1991; Thissen et al., 1994] and by increasing IGF-I metabolic clearance rate [Thissen et al., 1992]. Furthermore, the levels of liver IGF-I mRNA are reduced by protein restriction through pretranslational and translational defects [Thissen et al., 1991]. This is in keeping with the present results showing that pair-fed rats exhibited a marked decrease in circulating IGF-I levels compared with control rats. It is likely that a deficiency in IGF-I could be responsible, at least in part, for the reduction in skeletal longitudinal growth detected in TRP-free diet animals. The more pronounced reduction in IGF-I levels detected in TRP-free diet rats than in pair-fed group, could be explained by the evidence that TRP is an essential amino acid. Thus, a reduction in the hepatic supply of TRP could have affected more markedly IGF-I production resulting in a drastic reduced bone gain. Indeed, culture medium deficient in TRP is associated with a selective reduction of IGF-I synthesis by cultured rat hepatocytes [Harp et al., 1991].

Osteogenic cells are not only equipped with specific IGF-I receptors, but are also able to synthesize IGF-I, indicating an autocrine and/or paracrine action of IGF-I in the control of osteoblastic activity. [Giustina et al., 2008].

The possible influence of a TRP-free diet on the local production of IGF-I by osteoblasts remains to be clarified. However, a possible influence of local environment in protein or amino acid on IGF-I production has been previously described in mouse osteoblastic cells [Chevalley et al., 1996].

We are aware of some limitations of this study. First histomorphometric analysis could be useful to confirm the biochemical data on the effects of TRP deficiency on bone accrual. Secondly, the number of our experimental data did not allow us to perform a multivariate regression analysis to examine the relative contribution of a reduction of body weight or IGF-I levels on bone parameters detected in TRP-free diet rats.

In conclusion, this study has demonstrated that TRP deficiency impairs skeletal modeling and decreases bone mass in growing rats probably due to reduced 5-HT synthesis. This negative bone effect is associated with a negative shift in the balance between bone formation and resorption with a significant decrease in bone formation as evidenced by a reduction both in osteocalcin and IGF-I levels. These results extend our knowledge on the participation of 5-HT system in the neuronal regulation of growing bone and could be of interest in light of the evidence that childhood and adolescence, characterized by a rapid bone accrual may be times of particular vulnerability to the adverse effect of depression on bone.

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