The effect of chemical sympathectomy and stress on bone remodeling in adult rats

Kaijin Hu, Hongzhi Zhou, Guoliang Zhang, Ruifeng Qin, Rui Hou, Liang Kong, Yuxiang Ding

Department of Oral surgery, School of Stomatology, Fourth Military Medical University, P.R. China.

Correspondence to: Yuxiang Ding, MD.
Department of Oral Surgery, School of Stomatology, FMMU,
145 Western Changle Road, Xi'an, 710032, P.R. China.
TEL: +86-029-84776102; FAX: +86-029-84776026; E-MAIL: yxding@fmmu.edu.cn

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Abstract

OBJECTIVES: Bone remodeling has recently been revealed to be under sympathetic nerve control. The role of the sympathetic nerve system is not clearly understood. The present study aim to explore the effect of chemical sympathectomy and stress on bone remodeling in adult rats.

METHODS: 24 twelve-month-old Wistar rats were divided into three group (sympathectomy, stress and control). The sympathectomy and stress group rats were administered 6-hydroxydopamine (150 mg/kg each day) and saline (1 ml/kg each day) intraperitoneal respectively for one week and exposed to stress procedure for another three weeks. The stress procedure was mild, unpredictable footshock, administered for one hour once daily. Analysis of serum chemistry, microcomputed tomography, dual energy X-ray absorptiometry, biomechanical testing and bone histomorphometry were employed.

RESULTS: The stress group rats showed increased bone resorption in contrast to the sympathectomy and control group rats. The serum level of calcium and phosphorus cations and norepinephrine were enhanced, the cancellous bone volume and bone mineral density were reduced, bone mechanical property such as strength, ductility and toughness were weakened, the osteoclast counts and osteoclast surfaces were increased and the bone formatin rate were decreased significantly in the stress group rats in contrast to the other two groups rats. There was no significant difference of bone remodeling between the sympathectomy group and control group rats.

CONCLUSION: Our study showed stress-increased sympathetic nerve system activity enhanced bone resorption while chemical sympathectomy inhibited bone resorption under stress. We postulate sympathetic neurotransmitter and neuropepitide may play a role in regulating bone remodeling.

Abbreviations:

SNS	- sympathetic nerve system
μCT	- microcomputed tomography
DEXA	- dual energy X-ray absorptiometry
BMD	- bone mineral density
BFR	- bone formatin rate
ARs	- adrenergic receptors
NE	- norepinephrine
6-OHDA	- 6-hydroxydopamine
ALP	- alkaline phosphatase
TRAP	- tartrate-resistant acid phosphatase
Oc.N	- number of osteoclasts
BS	- bone surface
Oc.S	- osteoclast surface
CGRP	- calcitonin gene-related peptide
SP	- substance P
VIP	- vasoactive intestinal polypeptide
IR	- immunoreactive
NPY	- neuropeptide Y
DβH	- dopamine-β-hydroxylase
TH	- tyrosine hydroxylase

ORIGINAL ARTICLE

INTRODUCTION

Bone is continuously remodeled and regulated by various systemic and local factors, and numerous studies have demonstrated that bone metabolism can be influenced by the nervous system. Bone and periosteum are innervated by both autonomic and sensory nerves. Autonomic nerve fibers are found in the periosteum, endosteum, and cortical bone and, in many cases, the free-running fibers are associated with blood vessels that entered the bone through Volkmann's canals (Bjurholm *et al.* 1988 ; Fristad *et al.* 1994).

Nerve endings have been found to be in direct contact with bone cells, and catecholamine-containing axons have been identified near osteoblasts in vivo (Serre *et al.* 1999), suggesting a neuroendocrine regulation of bone remodeling. It has become evident that bone cells are equipped with functional receptors for several neuro-osteogenic factors (Togari *et al.* 1997; Kellenberger *et al.* 1998; Togari *et al.* 2000; Togari 2002; Lerner 2002), human osteoblastic as well as osteoclastic cells are equipped with adrenergic receptors (ARs) and neuropeptide receptors (Togari 2002; Lerner 2002).

The peripheral sympathetic nervous targets receive signals from the proximal upper nervous ending, which releases noradrenalin as a neurotransmitter into the synaptic gap. The extension of axons of sympathetic neurons to osteoblastic and osteoclastic cells is responsible for the dynamic neural regulation of local bone metabolism.

Ageing in humans is associated with increasing sympathetic nerve system (SNS) activity (Esler *et al.* 1995; Seals &. Esler 2005). Many old people are sufferring from anxiety, depression and insomnia oweing to increased SNS activity. In obese, ageing, postmenopausal women, there may be greater activation of the SNS than in lean ageing women (Sakhaee *et al.* 1985). Whether or not the enhanced SNS activity affect the bone remodeling is unknown.

Norepinephrine (NE) has been shown to increase bone resorption in bone organ culture, and propranolol (β-adrenergic receptor antagonist) to have the opposite effect (Moore *et al.* 1993; Dietrich *et al.* 1979). These findings suggest that the SNS mainly impacts on bone resorption. The SNS plays a negative regulatory role in bone formation and positively regulates bone resorption by stimulating osteoclastic differentiation, increase osteoclast numbers and activity.

6-hydroxydopamine (6-OHDA) is a neurotoxin used by neurobiologists to selectively kill dopaminergic and noradrenergic neurons. 6-OHDA enters the neurons via the dopamine and noradrenaline (norepinephrine) reuptake transporters. Most postganglionic sympathetic fibers release NE, they are noradrenergic fibers running to the tissues innervated. Stress can induce densification of sympathetic innervation by increasing SNS activity. The aim of the present study was to evaluate the effect of increased sympathetic activity under chronic stress and chemical sympathectomy by 6-OHDA on the bone remodeling in adult rats.

MATERIAL AND METHODS

Animals

Twenty four 12-month-old male Wistar rats weighing 400±30 g were used in the present study and treated in accordance with the Guidelines for Animal Experiments at the School of Stomatology, Fourth Military Medical University. Rats were divided to three group (sympathectomy, stress and control), eight for each group. Four rats of each group were caged together under automatically controlled conditions of temperature (23±1 °C), humidity (50±10%), and a 12: 12h light-dark cycle and were given ad libitum tap water and rodent chow.

Experimental protocol

6-OHDA (Sigma) was dissolved in saline and the concentration was 150 mg/ml. The injection were freshly prepared just before treatment, the sympathectomy group rats were injected intraperitoneal (0.1 ml/100 g) with 6-OHDA each day for one week, the stress group rats were injected saline (0.1 ml/100 g) with the same injection protocol. The sympathectomy and stress group rats were exposed to stress procedure for another three weeks. The stress procedure was mild, unpredictable footshock. The rats were subjected to unpredictable footshock (2 mA) through a grid floor once daily for one hou for 21 days. The duration of each shock and the interval between the shocks were randomly programmed between 3–5 and 10–100 sec, respectively (Bhattacharya & Muruganandam 2003). On day 21, all the rats were sacrificed by drawing blood from the abdominal arota by a heparinzed syringe under ether anesthesia and the femur, tibiae and lumber were harvested. 13 and 3 days before sacrifice, all the rats were injected intraperitoneally with tetracycline and calcein respectively.

Serum chemistry

The rats were anesthetized with ether, 3–5 ml of blood was withdrawn by abdominal aorta puncture and stored in heparinized centrifuge tubes. The blood sample was centrifuged and the supernatant was taken and stored at −70 °C. The serum level of calcium and phosphorus cations and alkaline phosphatase (ALP) were measured by biochemistry autoanalyzer (Hitachi 7180, Japan). The serum level of NE was measured using rat NE Elisa Kit (EHSY lab, China).

Analysis of trabecular structure by microcomputed tomography(μCT)

The proximal region of left tibia (stored in 70% ethanol) were subjected to three-dimensional μCT analysis using a μCT scanner (SkyScan 1174, Belgium). Scanning was initiated 1.5mm below the proximal tibiae growth plate, and a total of 100 consecutive 50 μm-thick sections were

analyzed, encompassing a 5 mm length of the secondary spongiosa. Cortical bone was excluded with semi-automatically drawn contours. Relative bone volume (BV/TV), trabecular number (Tb.N), thickness (Tb. Th), and separation (Tb.Sp) were calculated by measuring 3D distances directly in the trabecular network and taking the mean over all voxels.

Measurement of bone mineral density (BMD) by dual energy X-ray absorptiometry (DEXA)

After μCT scan, BMD of the proximal of left tibia was measured by dual-energy x-ray absorptiometry (Prodigy, GE Lunar Company). We set manually the region of interest (ROI) 3×3mm^2 area 1.5mm below the proximal tibiae growth plate. The BMD (mg/cm^2) of the ROI was obtained automatically.

Biomechanical testing

After sacrifice, the left femur and the second lumbar (L2) were immediately dissected free and cleaned by removing the flesh, stored in saline at –20 °C. Bone samples were thawed before biomechanical testing. The femur was used for 3-point bending test while the trunk of lumbar was separated and made into column contour for compressing test. The biomechanical test was performed in a servohydraulic materials testing system (MTS858, MN, USA). The femur was positioned on the top of two separate metal rods which the distance between two rods was 20 mm. The striker pressed the midshaft of the femur at a constant displacement rate of 10mm/min until the bone was broken. The lumbar were axially compressed at a constant displacement rate of 1mm/min until the bone was squeezed or broken. Both displacement and load were recorded for later analysis. Ultimate force (Strength) was determined from the load-displacement curves, and ultimate stress was calculated by dividing the ultimate force by the cross-sectional area.

Bone histomorphometry

The right proximal tibia were fixed in 4% paraformaldehyde for 48 hours and decalcified in 20% EDTA for three weeks. The specimens were dehydrated in ethanol and embedded in paraffin, sectioned longitudinally at 4 μm thick, stained for tartrate-resistant acid phosphatase (TRAP). TRAP-positive multinucleated cells (red stained) attached to bone were scored as osteoclasts. Measurements were made within an area of 0.8 mm^2 (1.0 × 0.8 mm), with its closest and furthest edges at 2.0 and 3.0 mm distal to the growth plate of the proximal ends of the tibia. Histomorphometry was conducted to quantify the number of osteoclasts (Oc.N/BS) and osteoclast surface (Oc.S/BS) as defined by Parfitt *et al.* (1987). The right distal femur were dehydrated in ethanol and embedded without demineralization in methylmethacrylate, sectioned longitudinally at 8 μm thick unstained for measurements of fluorochrome-based indices. Interlabel width between the double labels tetracycline (green) and calcein (yellow) were measured and the bone formation rate (BMR) were calculated.

Statistical analysis

All data were presented as Means ± SEM. Statistical analysis was carried out by one-way ANOVA.

RESULTS

Serum chemistry

As Table 1 shown, the serum level of NE, calcium and phosphorus was increased significantly by 106.4% (p<0.01), 14.5% (p<0.05) and 15.2% (p<0.05) respectively and the level of ALP was decreased by 10.9% (p>0.05) in the stress group rats compared to the control group rats. There was no significant difference in serum chemistry between the sympathectomy and control group rats.

Analysis of trabecular structure and BMD

Two-dimension and three-dimension μCT image showed the trabecular bone volume decreased and separation increased in the proximal tibia in stress group rats compared to the other two group rats (Figure 1). The effect of chemical sympathectomy and stress on the trabecular structure and BMD in the proximal tibia was shown on Table 2. BV/TV was reduced 26.1% (p<0.05), Tb.Sp was increased 18.2% (p<0.05) and BMD was decreased by 23.1% (p<0.01) in the stress group rats compared to the control group rats.

Biomechanical property measured by mechanical test

Lumber (L2) compressing test showed the bone mechanical property parameters such as strength, ductility and toughness were decreased by 23.6% (p<0.05), 13.9% (p<0.05) and 33.9% (p<0.01) respectively in the

Tab. 1. Measurement of serum chemistry.

Group	NE (ng/L)	Calcium (mmol/L)	Phosphorus (mmol/L)	ALP (U/L)
Control	47±5.3	2.35±0.27	1.58±0.19	128±15.2
Sympathectomy	34±3.6	2.42±0.30	1.63±0.22	123±13.7
Stress	97±8.2**	2.69±0.43*	1.82±0.25*	114±10.1

Values represent Means±SEM. (n=8); * p<0.05, ** p<0.01 statistical difference from control by one-way ANOVA.

Tab. 2. Measurement of proximal tibia trabecular bone structure and BMD.

GROUP	BMD (mg/cm^2)	BV/TV (%)	Tb.Th (μm)	Tb.N (1/mm)	Tb.Sp (μm)
Control	230±13	25.3±0.7	137±1.9	0.787±0.06	286±9.0
Sympathectomy	223±15	24.6±0.8	136±1.0	0.775±0.09	301±11.7
Stress	177±17**	18.7±1.1*	127±1.3	0.599±0.08	338±12.0*

Values represent Means±SEM. (n=8); * p<0.05; ** p<0.01 statistical difference from control by one-way ANOVA.

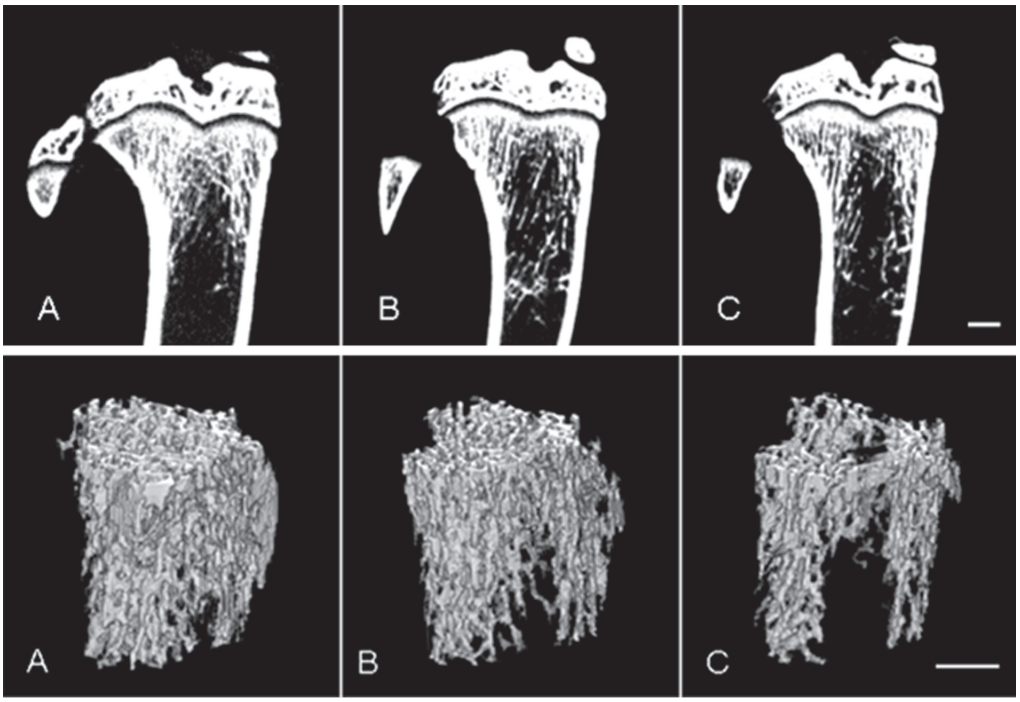

Fig 1. Representative two and three-dimensional μCT images of trabecular microarchitecture of the proximal tibia. (A) control group, (B) sympathectomy group, (C) stress group. Bar=1mm. μCT image showed the trabecular bone volume decreased and separation increased in stress group rats compared to the other two group rats.

Tab. 3. Mechanical properties of the lumber 2 by compressing test.

Group	Max force "Strength" (N)	Max deflection "Ductility" (mm)	Area Toughness (mJ)	Yield Modulus of elasticity (N/mm^2)
Contrl	267±7.3	0.909± 0.070	123.6±16.9	224.4±10.2
Sympathectomy	271±6.5	0.912± 0.056	124.2±15.7	226.6±12.3
Stress	204±5.6*	0.783±0.035*	81.8±12.9*	211.1±10.3

Values represent Means±SEM. (n=8); * $p<0.05$ statistical difference from control by one-way ANOVA.

Tab. 4. Mechanical properties of the femur by 3-point bending test.

Group	Max force "Strength" (N)	Max deflection "Ductility" (mm)	Area Toughness (mJ)	Yield Modulus of elasticity (N/mm^2)
Control	138.2±3.4	0.931±0.036	88.9±5.1	284.4±6.8
Sympathectomy	133.2±4.5	0.925±0.024	84.9±3.9	280.4±4.2
Stress	129.8±2.8	0.919±0.018	85.8±4.0	281.1±5.0

Values represent Means±SEM. (n=8); No significant differences were observed from the control by one-way ANOVA.

stress group rats in contrast to the control group rats (Table 3).

In femur midshaft 3-point bending test, all of the mechanical properties (strength, stiffness, ductility and toughness) had no significant difference between the three groups (Table 4).

Bone histomorphometry

Figures 2 and 3 showed representative TRAP stained proximal tibia sections and uncalcified double-labelling distal femur sections respectively. TRAP-positive red stained osteoclasts were seen increased in the stress group rats. The number of osteoclasts (Oc.N/BS) and the osteoclast surface (Oc.S/BS) were increased by 91.2% ($p<0.01$) and 45.6% ($p<0.01$) respectively while the bone formation rate was decreased by 46.6% ($p<0.05$) in the stress group rats compared to the control group rats (Figure 4).

DISCUSSION

In this study, we found the skeletal structure of adult rats was destructed by increased SNS activity under stress. The higher serum norepinephrine concentration suggestsed a higher SNS activity in stress group rats than in control rats. Trabecular volume and thickness of proximal tibia was reduced and trabecular separation was increased in the stress group rats, the biomechanical property such as strength, ductility and toughness were also reduced in the trunk of lumbar 2, which indicated stress induced trabecular resorption and perforation and bone mechanical quality decline. Bone histomorphometry evidence of increased osteoclast number and surface and decreased bone formation rate supported the results above.

While the stress-enhanced bone resorption was inhibited by 6-OHDA induced chemical sympathec-

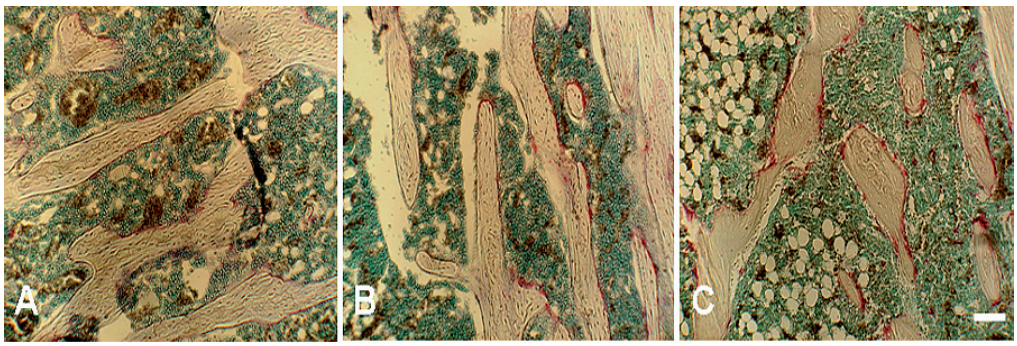

Fig 2. Representative microscopic photographs of TRAP-stained histological sections of the trabecular bone. Osteoclast was labelled as red-stained cell. (A) control group, (B) sympathectomy group, (C) stress group. Original amplfication 10×10, Bar=100 μm. The number of osteoclasts (Oc.N/BS) and the osteoclast surface (Oc.S/BS) were increased in the stress group rats in contrast to other two group rats.

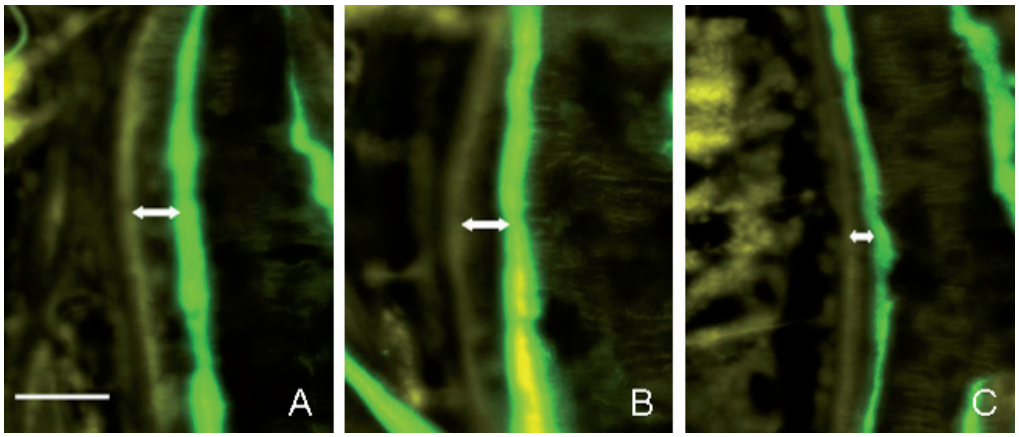

Fig 3. Representative fluoroscopic photographs of uncalcified double-labelling (tetracycline and calcein) histological section of of the trabecular bone. (A) control group, (B) sympathectomy group, (C) stress group. Original amplfication 10×20, Bar=100 μm. Interlabel width was decreased in the stress group rats in contrast to other two group rats.

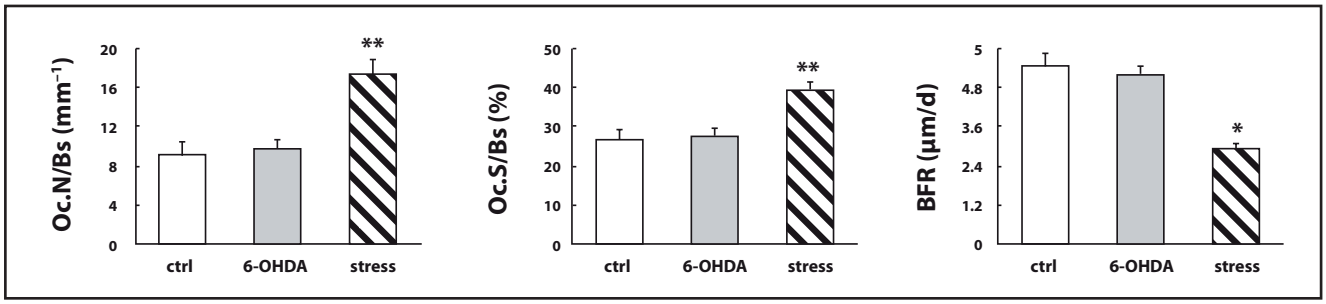

Fig 4. Histomorphometry analysis in the distal femur. The number of osteoclasts (Oc.N/BS) and the osteoclast surface (Oc.S/BS) were increased by 91.2% ($p<0.01$) and 45.6% ($p<0.01$) respectively while the bone formation rate (BFR) was decreased by 46.6% ($p<0.05$) in the stress group rats compared to the control group rats. BFR = measurement of interlabel width/ interval days.

tomy. Our results was contrary to the study of the Cherruau *et al.* (1999) who reported chemical sympathectomy either by guanethidine or by 6-OHDA impaired bone resorption in rats. 6-OHDA is a sympatholytic agent with direct and specific toxic effects on terminal sympathetic fibers. 6-OHDA is specifically taken up by sympathetic neurons via their membrane amine pumps (Lerner *et al.* 1994) and stored within neuronal catecholamine storage granules as a "false neurotransmitter" thus rapidly displacing norepinephrine from vesicles and depleting terminals of endogenous catecholamine (Lerner *et al.* 1994; Lundberg *et al.* 1996) leading to chemical sympathectomy.

The regulation of bone mass under SNS control has been demonstrated both pharmacologically and genetically. Recent studies in genetically altered mice have established that the enhanced SNS activity treated with β-agonist isoproterenol or clenbuterol played a negative regulatory role in bone formation and positively regulated bone resorption by stimulating osteoclastic differentiation (Takeuchi *et al.* 2001; Takeda *et al.* 2002; Harada & Rodan 2003; Bonnet *et al.* 2005).

While lowered sympathetic tone in mice treated with the β-blocker propranolol, mice deficient for dopamine β-hydroxylase (the step-limiting enzyme responsible for catecholamine synthesis), and leptin-deficient Ob/Ob mice (Elefteriou *et al.* 2005a,b) induced increased osteoblast number and activity and a subsequent increase in bone mass.

As described above, our study indicated that sympathetic innervation contribute to the skeletal homeostasis. The enhanced SNS activty under stress induced bone resorption trabecular loss and biomechanical property decline while the chemical sympathectomy by 6-OHDA treatment inhibit bone resorption under stress circumstance. What is the underlying mechanism? The enhanced SNS activity stimulated by stress triggered the β2-adrenergic activity in both osteoblastic and osteoclastic cells. β-agonists have been shown to stimulate production of bone-active cytokines (*e.g.* IL-6, IL-11, and prostaglandin E2), PTH and RANKL (Kondo *et al.* 2001; Takeuchi *et al.* 2001; Kondo & Togari 2003; Schmitt *et al.* 2003) and to increase osteoclastogenesis. Elevated SNS tone positively regulated bone resorption by stimulating osteoclastic differentiation, increased osteoclast numbers and activity. The down regulation of bone formation was also dependent on the activation of β2 adrenergic receptors, expressed by osteoblasts. Adrenergic stimuli, enhance osteoclastic differentiation and inhibit osteoblasts functions, resulting in a negative bone mineral balance. The sympathetic nervous system mainly impacted on bone resorption. Lowered SNS tone by chemical sympathectomy under 6-OHDA treatment reversed the negative bone remodeling effect induced by stress stimuli.

Development and differentiation of osteoblasts and osteoclasts are locally controlled by growth factors and cytokines produced in the bone marrow microenvironment. Signals derived from the endocrine and autonomic nervous systems also exert potent effects on osteoclast and osteoblast development and differentiation. In fact, a ubiquitous autonomic innervation of all periosteal surfaces exists and its disruption may affect bone remodeling control. Bone envelopes are innervated by both myelinated and unmyelinated fibers belonging to the sensory and sympathetic nervous systems, releasing sensory [calcitonin gene-related peptide (CGRP), and substance P (SP)] and sympathetic [vasoactive intestinal polypeptide (VIP), and neuropeptide Y (NPY)] neuropeptides. We postulated that these neurotransmitters may play a role in bone remodeling. It was well documented with the observation that sympathectomy induces sensory hyperinnervation (Schon *et al.* 1985; Aberdeen *et al.* 1990; Donnerer *et al.* 1991; Mione *et al.* 1992). This may have resulted in an increase in tissue sensory fibers, and ultimately in neuromediators (particularly CGRP).

In vitro, recent studies showed some of these neuropeptides modulate osteoclastic bone resorption; for example, VIP activates bone resorption (Hohmann *et al.* 1983; Lundberg *et al.* 1996), while CGRP inhibits it (D'Souza *et al.* 1986; Zaidi *et al.* 1987b; Akopian *et al.* 2000; Ishizuka *et al.* 2005). Moreover, administration of CGRP partially inhibits bone loss in ovariectomized rats (Valentijn *et al.* 1997).

In addition, Blood flow is controlled largely by neurogenic mechanisms, and several neuropeptides have vasoregulatory activity. Recent studies (Bjurholm *et al.* 1988; Hill & Elde 1991; Ahmed *et al.* 1993) showed the association of nerve fibers with the vascular elements entering Volkmann's canals demonstrates that the interior of bone, like the periosteum, is well-innervated. With the use of immunohistochemistry, it was demonstrated that VIP-immunoreactive (IR) nerve fibers were spicuously absent from these vascular channels, while NPY-, dopamine-β-hydroxylase (DβH) and tyrosine hydroxylase (TH)-IR fibers were predominantly found in the blood vessels walls, indicating the sympathetic origin of these nerves were involved in modulating blood-flow through bone.

NPY potentiates the vasoconstrictive action of noradrenaline (McDonald 1988), NPY and NE caused elevation of the perfusion pressure and decline in intraosseous pressure, which was evidence of intraosseous vasoconstriction (Lindblad *et al.* 1994). NPY with NE, acts as a sympathetic neurotransmitter in the control of vascular tone in bone. On the other hand, CGRP is a potent vasodilator (Brain *et al.* 1985; Zaidi *et al.* 1987a; Lundgaard *et al.* 1997). The enhanced SNS activity under stress induced decreased blood-flow through bone by releasing NPY and NE neurotransmitter, causing to disruption or alteration in the trophic relationship between sympathetic fibers and their end-organ bone, which may play a role in the activation of bone resorption. While chemical sympathectomy under 6-OHDA treatment depleted NPY-IR and cetecholaminergic fibers, moreover the CGRP-IR nerves were increased instead. CGRP-IR fibers hyperinnervation may counteract the negative regulation of blood-flow and nutrient delivery to bone by the enhanced sympathetic tone under stress.

In conclusion: The present study showed stress-increased SNS activity enhanced bone resorption. While chemical sympathectomy by 6-OHDA treatment inhibited bone resorption under stress. We postulate stress-induced trabecular bone loss and biomechanical property decline is mediated by releasing local sympathetic neurotransmitter and regulating blood-flow to bone.

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