

Effects of nicotine on bone and calciotropic hormones in aged ovariectomized rats

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Abstract

The objective of this investigation was to assess the effects of chronic nicotine administration on bone status and serum calcium and calciotropic hormone levels in aged, estrogen-replete (intact, sham-operated) and estrogen-deplete (ovariectomized) female rats. Eight-month-old sham-operated (sham) and ovariectomized (ovx) retired breeder rats were maintained untreated for 3 months to allow for the development of osteopenia in the ovx group. The animals were then administered either saline, low dose nicotine (6.0 mg/kg/day), or high dose nicotine (9.0 mg/kg/day) via osmotic minipumps for 3 months. Blood was drawn at necropsy for determination of serum nicotine, cotinine, Ca, PTH, 25(OH)D, and 1,25(OH)₂D. Right tibiae were collected and processed undecalcified for cancellous and cortical bone histomorphometry. Histomorphometric endpoints evaluated at the proximal tibial metaphysis included cancellous bone volume (BV/TV), osteoclast surface (Oc.S), osteoid surface (OS), mineralizing surface (MS), mineral apposition rate (MAR), and bone formation rate (BFR). Histomorphometric endpoints evaluated at the tibial diaphysis included cortical area (Ct.Ar), marrow area (Ma.Ar), and periosteal and endocortical MS, MAR, and BFR. Ovariectomy resulted in lower cancellous BV/TV and Ct.Ar and higher cancellous, endocortical, and periosteal MS and BFR. The presence of nicotine in serum confirmed successful delivery of the drug via osmotic minipumps. Administration of nicotine at the high dose resulted in lower serum 25(OH)D levels but differences in serum Ca or PTH were not detected with either nicotine treatment. Differences with nicotine treatment were also not detected for Oc.S at the proximal tibia. While treatment with nicotine at the high dose resulted in higher MS and BFR, in both sham and ovx rats, there were no differences due to nicotine treatment in cancellous BV/TV. Marrow area was greater in rats treated with nicotine than in rats treated with vehicle. However, differences with nicotine treatment were not detected in Ct.Ar in either intact or ovx rats. Overall, these findings indicate that steady state nicotine exposure does not alter bone mass in intact or ovx rats but may have detrimental effects on body storage of vitamin D.

Keywords: Nicotine, Bone Histomorphometry, Osteopenia, Rats

Introduction

Postmenopausal osteoporosis is a heterogeneous disease of skeletal fragility that leads to increased fracture risk¹. Heredity, hormonal status, age, and various environmental factors exert modulating effects on bone and contribute to the etiology of this condition. Smoking is a widely implicated life-style risk factor for osteoporosis. Numerous epidemiological and clinical studies have found some degree of asso-

ciation between smoking, reduced bone mass, and increased fracture risk². Among women, this association is observed predominantly at or after menopause^{3,4} suggesting a cumulative lifetime effect of smoking on bone or greater resistance to smoking by a younger, estrogen-replete skeleton.

Although compelling, the evidence for the negative association between smoking and bone health is based predominantly on epidemiological studies, the limitations of which include difficulty in controlling for confounding variables such as nutrient intake⁵, body weight⁶, and alcohol consumption². Use of animal models can circumvent some limitations of human studies since confounding variables can be eliminated or minimized, which allows the investigators to study the direct effects of tobacco on bone health. In addition, specific components of tobacco can be assessed separately in

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Week	Procedure			
-12	Eight-month-old, retired breeder rats left untreated to establish cancellous osteopenia in the OVX group			
-3	Rats randomized by weight into four treatment groups			
		Treatment	Nicotine Dose	Sham (n) Ovx (n)
		Baseline	None	14 14
		Vehicle	0 mg/kg/day	17 16
		Low Dose Nicotine	6 mg/kg/day	16 16
		High Dose Nicotine	9 mg/kg/day	22 22
-1	Bone specimen collection from baseline control groups			
0	Start of treatment, osmotic minipumps implanted in all rats			
4	Minipumps replaced			
8	Minipumps replaced			
12	Bone specimen collection from treatment groups			
		Treatment	Nicotine Dose	Sham (n) Ovx (n)
		Vehicle	0 mg/kg/day	15 ^a 15 ^a
		Low Dose Nicotine	6 mg/kg/day	15 ^a 16
		High Dose Nicotine	9 mg/kg/day	17 ^{ab} 19 ^{ab}

^aRats died during surgery (n=1/group except for Sham Vehicle where n=2).
^bRats died 1-12 days following surgery (n=4 for Sham and 2 for OVX).

Table 1. Experimental protocol.

animal models so as to determine which components are most likely responsible for the deleterious effects of smoking on bone⁷⁻¹⁷. Despite these advantages, animal models have been underutilized for studying the skeletal effects of tobacco in general and during estrogen depletion in particular. Therefore, the current investigation was designed to evaluate the effects of nicotine, the principal and addictive alkaloid in tobacco, on cancellous and cortical bone and on serum mineral and calcitropic hormone levels in aged, estrogen-replete and estrogen-deplete female rats.

Materials and methods

One hundred and forty, approximately eight-month-old, retired breeder, sham-operated (sham, n=70) and ovariectomized (ovx, n=70), female Sprague Dawley rats (Harlan, IN) were used in the experiment, which was run as two repeats (group 1 and group 2) of 70 rats each (35 sham and 35 ovx) to facilitate animal handling and sample collection. The animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the

experimental protocol was approved by the Creighton University Animal Resource Facility. The rats were housed individually in hanging wire cages and fed a laboratory rat diet (Laboratory Rodent Diet 5001, Purina Mills) with a calcium, phosphorus, and vitamin D content of 1%, 0.61%, and 4.5%, respectively. Food and water were provided *ad libitum* to the sham rats. Food consumption of the ovx rats was restricted to that of the sham control rats.

Experimental protocol

The rats were left untreated for 12 weeks after surgery (week -12 to 0) to establish cancellous osteopenia in the ovx group (Table 1). Three weeks prior to nicotine treatment, the rats were randomized by weight into four sham or ovx treatment groups: baseline, vehicle, low dose nicotine, and high dose nicotine. Bone tissue from the baseline groups (sham and ovx) was collected one week prior to initiation of nicotine treatment. Nicotine and vehicle were administered from week 0 to week 12 using Alzet osmotic minipumps model 2004 (Alza Corp., Palo Alto, CA). This minipump

delivers solutions at a rate of 0.25 $\mu\text{l/hr}$ for up to 32 days. Nicotine tartrate (Sigma Chemical Co., St. Louis, MO) was dissolved with ultrasound in sterile saline for an average delivery of 6.0 mg nicotine/kg/d (low dose) or 9.0 mg nicotine/kg/d (high dose). Control animals received saline. The minipumps (2/rat) were implanted subcutaneously posterior to the shoulder under ketamine (50 mg/kg) and xylazine (10 mg/kg) anesthesia administered by intraperitoneal injection. The minipumps were replaced every 4 weeks for the 3-month duration of treatment. Minipumps were used to eliminate stress associated with multiple daily injections and circumvented the large instantaneous burst of nicotine to the experimental animal.

A double fluorochrome labeling technique was used to determine active mineralization sites and rates of bone formation. Rats were injected subcutaneously with calcein (7 mg/kg) (Sigma Chemical Co., St. Louis, MO) at 10 and 3 days prior to necropsy. For sample collection, rats were anesthetized with ketamine/xylazine as described above. Blood was drawn via cardiac puncture and serum specimens stored at -70°C until assay. Successful ovariectomy was confirmed in all ovx animals by observation of lack of ovarian tissue and atrophied uterine horns. Tibiae were excised, cleaned of soft tissue, and the tibial anterior eminence was scraped to expose bone marrow. The tibiae were then fixed in 70% ethanol for histologic processing.

Serum analysis

The following serum endpoints were determined: nicotine and cotinine by gas chromatography (detection limits, 0.5 ng/ml for nicotine and 5 ng/ml for cotinine) (Clinical Pharmacology Laboratories, University of California-San Francisco);¹⁸ calcium by the o-cresolphthalein compliance method (Roche Cobas Integra model 700); PTH by a rat PTH immunoradiometric assay (IRMA) kit (detection limit, 1 pg/ml) from Immunotopics (San Clemente, CA); 25(OH)D by an assay kit (detection limit, 2.2 ng/ml) from Quest Diagnostics Nichols Institute (San Juan Capistrano, CA); and 1,25(OH)₂D by a radioimmunoassay (RIA) kit (detection limit, 2.0 pg/ml) from Diasorin Inc. (Stillwater, MN).

Bone histomorphometry

The right tibiae were cut 1 mm distal to the tibio-fibular junction (TFJ) and 19 mm proximal to the TFJ to obtain specimens from the proximal metaphysis and cortical diaphysis. The specimens were then placed in Villanueva stain¹⁹ for 3 days, dehydrated in graded ethanols and acetone, and embedded in modified methyl methacrylate²⁰. Histomorphometric cancellous and cortical bone data were collected with a light/epifluorescent microscope and a video camera interfaced with BIOQUANT TCW software (R&M Biometrics, Nashville, TN). Histomorphometric data are reported in accordance with standard bone nomenclature²¹.

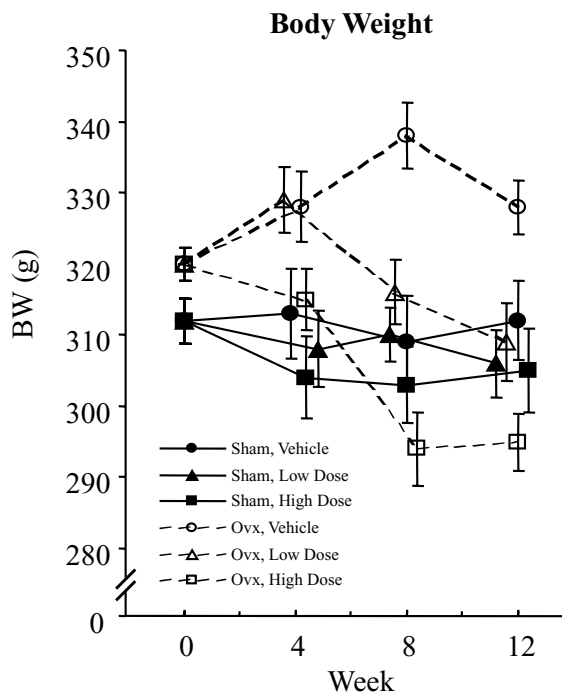


Figure 1. Body weight (mean \pm SE) at commencement of nicotine treatment (weeks 0), pump re-implantation (weeks 4 and 8), and termination of nicotine treatment (week 12).

For cancellous bone histomorphometry, frontal sections (5 μm thick) were cut from the central third of each proximal tibia with a vertical bed microtome (Jung Supercut 2050) and affixed to slides. One slide was stained by the Goldner method²² and the second slide was coverslipped without further staining. The Goldner-stained specimens were used for determining structural and static endpoints and the Villanueva-stained specimens were used for assessing fluorochrome labeling and dynamic measurements of bone formation. For data collection, a measurement area (approximately 5 mm²) was outlined 1 mm distal to the growth plate cartilage to include secondary spongiosa only. The following measurements were made: at 20X, total tissue area (Tt.Ar); at 100X, total trabecular area (Tb.Ar) and perimeter (Tb.Pm); at 160X, single- and double-labeled perimeter (sL.Pm and dL.Pm); and at 400X, the interlabel width of double labels (Ir.L.Wi), osteoid perimeter (O.Pm), and osteoclast perimeter (Oc.Pm). The following calculations were made: cancellous bone volume (BV=Tb.Ar/Tt.Ar), osteoid surface [OS=(O.Pm/Tb.Pm) x 100], osteoclast surface [Oc.S=(Oc.Pm/Tb.Pm) x 100], single-labeled surface [sLS=(sL.Pm/Tb.Pm) x 100], double-labeled surface [dLS=(dL.Pm/Tb.Pm) x 100], mineralizing surface (MS=dLS+0.5sLS), mineral apposition rate [MAR=Ir.L.Wi/Ir.L.t (interlabel time period)], and annual bone formation rate (BFR=MS x MAR x 365 days).

For cortical bone histomorphometry, the embedded corti-

Endpoint	Sham-operated Rats			Ovx Rats			ANOVA Main Effects*		
	Vehicle	Low Dose Nicotine	High Dose Nicotine	Vehicle	Low Dose Nicotine	High Dose Nicotine	OVX P<	TRT P<	OVX x TRT P<
Nicotine (ng/ml)	2±1	115±12	229±23	<1	99±9	170±14	0.0033	0.0001 ^{a,b}	
Cotinine (ng/ml)	ND**	740±54	1054±57	ND**	706±63	978±65		0.0001 ^{a,b}	
Calcium (mg/dL)	10.5±0.1	10.4±0.1	10.3±0.1	10.2±0.1	10.2±0.1	10.2±0.1	0.0144		
PTH (pg/ml)	116±10	120±18	93±11	118±13	103±15	132±12			
25(OH)D (ng/ml)	44±2	44±2	34±2	38±2	39±1	36±2		0.0211 ^b	
1,25(OH) ₂ D (pg/ml)	30±3	30±3	20±2 ^{b*}	27±4	32±3	41±3 ^{b*}	0.0252		0.0051

Results are mean±SEM of 11-19 animals. Samples sizes differ based on serum availability and number of maximum samples analyzed per assay kit.

*OVX, ovariectomy effect; TRT, treatment effect; OVX x TRT, ovariectomy by treatment interaction

**Not detectable

^aLow dose nicotine different from vehicle, P<0.05

^bHigh dose nicotine different from vehicle, P<0.05, ^{b*}P<0.1

Table 2. Serum Chemistry in Aged Rats After 3 months of Nicotine Treatment.

cal samples were cross-sectioned at 80 µm thickness between 5 and 7 mm proximal to the TFJ on a saw microtome (Model 1600, Leica, Germany). One section was mounted on a glass slide with Permount. The following data were collected for each specimen: at 20X, total and marrow area (Tt.Ar, Ma.Ar), periosteal and endocortical bone perimeters (Ps.Pm and Ec.Pm); at 160X, single- and double-labeled perimeters (sL.Pm and dL.Pm); and at 400X, inter-label width (Ir.L.Wi) at sites of double labeling. The following calculations were made: cortical area (Ct.Ar=Tt.Ar-Ma.Ar), single-labeled surface [sLS=(sL.Pm/B.Pm) x 100], double-labeled surface [dLS=dL.Pm/B.Pm x 100], mineralizing surface (MS=dLS+0.5sLS), mineral apposition rate (MAR=Ir.L.Wi/Ir.L.t), and annual bone formation rate (BFR=MS x MAR x 365 days).

Statistical analysis

The effects of ovariectomy status (OVX, with two levels; sham and ovx), treatment (TRT, with three levels; vehicle, low dose nicotine, and high dose nicotine), repeat (with two levels; 1 and 2, experiment run as two repeats to facilitate animal handling and data collection), and their interaction (including OVX x TRT) on each dependent variable were analyzed. For histomorphometric data, rats sacrificed at baseline (11 months old) were included as a fourth level in the treatment group. A multi-way ANOVA was used as implemented in PROC GLM in the statistical package SAS 8.0. Variation was observed between repeats 1 and 2 and this was controlled for in the analysis. The data were trans-

formed when necessary to satisfy the ANOVA assumptions of normality and homogeneity of variance. Dunnett's test was used as a post-hoc test to compare low and high dose nicotine-treated rats to vehicle-treated rats whenever treatment had a statistically significant effect. When the interaction between ovariectomy status and treatment was significant, a separate analysis was conducted for each fixed level of one factor while varying the other factors. The level of statistical significance was set at 0.05 on all occasions, including the Dunnett's test.

Results

Death rate varied among treatment groups with 11 % of rats (15/140) dying before completion of the experiment (Table 1). Three rats died prior to randomization at 11 months of age (week -3) from what appeared to be natural causes. Six rats died while anesthetized for pump implant surgery. These deaths were not related to treatment or surgery. Six rats in the high dose nicotine group (4 sham and 2 ovx) died one to 12 days after pump implant surgery. These deaths occurred following the initial pump implantation in all but one case. Symptoms associated with the high dose nicotine treatment included shaking, lethargy, decreased grooming, decreased food consumption, and weight loss. Although some of these symptoms were, on occasion, observed in rats administered the lower nicotine dose, they did not result in death.

The implanted pumps were tolerated well by the majority of the animals. Thirteen percent of rats (13/97) showed tis-

sue inflammation and/or infection associated with the implant site. Of these, eight were in the high dose nicotine group (6 sham and 2 ovx), four were in the low dose nicotine group (2 sham and 2 ovx), and one was in the control group (ovx). When infection was observed, topical antibiotics were applied and pumps re-implanted within three days. Rat deaths did not appear to be associated with observable tissue infection.

Body weight did not differ between sham and ovx rats at the initiation of the study (week -12: sham, 309±4g; ovx, 303±3g). However, at the initiation of nicotine treatment (week 0), ovx rats weighed 3% more than sham rats ($P<0.04$). A negative, nicotine-dependent effect ($P<0.0001$) on body weight was observed after pump implant surgery in both sham and ovx rats. During the week following surgery, weight loss averaged 2.5% in the low dose nicotine group and 5.0% in the high dose nicotine group. Most of this weight was recovered by the second week post-surgery. Nicotine had a negative effect on body weight in both sham and ovx animals (Figure 1). During the 3 month duration of treatment (week 0-12), body weight increased by 1.4% in the vehicle-treated sham rats whereas it decreased by 2.2% and 1.6% in sham rats treated with low and high dose nicotine, respectively. In the ovx group, body weight increased by 3.0% in the vehicle treated rats, whereas it decreased by 5.4% and 6.6% in rats treated with low and high dose nicotine, respectively. The difference in body weight change between the low and high dose nicotine-treated rats was not significant regardless of estrogen status.

Serum chemistry

The effect of ovariectomy and nicotine treatment on serum chemistry is presented in Table 2. Ovx rats had lower serum nicotine and calcium and higher serum 1,25(OH)₂D levels than sham rats. Differences in serum cotinine, PTH, and 25(OH)D were not detected with ovariectomy.

Serum nicotine and cotinine were higher in the nicotine-treated rats than in the vehicle-treated rats, irrespective of estrogen status. Serum 25(OH)D was lower in both sham and ovx rats treated with high dose nicotine than in vehicle-treated rats. However, serum 1,25(OH)₂D showed a different response to nicotine treatment in the sham and ovx rats. In the sham group, rats treated with high dose nicotine tended to have lower ($P<0.1$) serum 1,25(OH)₂D levels than vehicle-treated rats while in the ovx group, rats treated with high dose nicotine tended to have higher ($P<0.1$) serum 1,25(OH)₂D levels than vehicle-treated rats. Significant differences in serum calcium and PTH were not detected with nicotine treatment.

Bone measurements

Cancellous bone volume at the proximal tibial metaphysis averaged 40% lower in ovx rats than in sham rats (Table 3). Osteoid surface, mineralizing surface, and bone formation

rate were greater in the ovx rats than in the sham rats. Differences in osteoclast surface and mineral apposition rate were not detected with ovariectomy.

Nicotine treatment had no effect on cancellous bone volume, osteoid surface, or osteoclast surface, irrespective of estrogen status (Table 3). Mineralizing surface varied with treatment and there was an interaction with ovariectomy. In the sham rats, mineralizing surface was greater ($P<0.05$) in rats treated with high dose nicotine and tended to be greater ($P<0.1$) in rats treated with low dose nicotine than in vehicle-treated rats. In the ovx rats, mineralizing surface was greater in rats treated with high dose nicotine than in the vehicle-treated rats and greater in baseline (11 month old) rats than in vehicle-treated (15 month old) rats. Mineral apposition rate did not differ with nicotine treatment but tended to be greater ($P<0.1$) in baseline rats than in vehicle-treated rats, irrespective of estrogen status. Bone formation rate varied with treatment and there was a marginal (<0.06) interaction with ovariectomy. In both the sham and the ovx animals, bone formation rate was greater in rats treated with high dose nicotine than in rats treated with vehicle. In the ovx rats, bone formation rate was also greater in baseline rats than in vehicle-treated rats. In the sham rats, this difference was only marginally significant.

The effects of ovx and nicotine treatment on cortical bone at the tibial diaphysis are presented in Table 4. Total tissue cross-sectional area was not affected by ovx, but cortical bone area was lower and marrow area was greater in the ovx rats than in the sham rats. In addition, mineralizing surface, mineral apposition rate, and bone formation rate at the periosteal and endocortical envelopes were greater in ovx rats than in sham rats.

Nicotine treatment had no effect on total tissue and cortical bone area. However, marrow area was greater in rats treated with high dose nicotine and tended to be greater ($P<0.1$) in rats treated with low dose nicotine than in the vehicle-treated rats, irrespective of estrogen status. Periosteal mineralizing surface varied with treatment and there was an interaction with ovariectomy. In sham rats, periosteal mineralizing surface was greater in rats treated with high dose and low dose nicotine than in vehicle-treated rats, while the only difference in ovx rats was greater mineralizing surface at baseline than after three months of vehicle administration. Periosteal mineral apposition rate and bone formation rate did not differ with nicotine treatment but were greater in baseline rats than in vehicle-treated rats in both the sham and the ovx groups. Endocortical mineralizing surface varied with treatment and there was a marginal ($P<0.09$) interaction with ovariectomy. Differences in endocortical mineralizing surface were not detected with treatment in sham rats while endocortical mineralizing surface tended to be lower ($P<0.1$) in baseline ovx rats than in vehicle-treated ovx rats. Although a treatment effect was detected for endocortical bone formation rate, baseline, low dose, or high dose nicotine-treated rats did not differ significantly from vehicle-treated rats.

Table 3
Histomorphometry of Proximal Tibia (Cancellous Bone) in Aged Rats After 3 Months of Nicotine Treatment

Endpoint	Sham-operated Rats				Ovx Rats				ANOVA Main Effects*			
	Basal	Vehicle	Low Dose Nicotine	High Dose Nicotine	Basal	Vehicle	Low Dose Nicotine	High Dose Nicotine	O VX P <	TR T P <	O V X x T R T P <	P <
Bone Volume (%)	5.1±0.8	6.8±0.9	7.3±0.8	5.0±0.8	3.2±0.4	2.1±0.3	3.5±0.6	2.3±0.3	0.0001			
Osteoid surface (%)	0.4±0.2	0.2±0.1	0.2±0.1	0.4±0.1	2.3±0.6	2.2±0.5	2.1±0.4	3.3±0.8	0.0001			
Osteoclast surface (%)	1.4±0.2	1.6±0.3	2.6±0.5	1.1±0.2	2.5±0.6	2.0±0.4	2.0±0.4	1.8±0.4				
Mineralizing surface (%)	4.4±0.6	3.3±0.3	5.2±0.6 ^{b*}	7.2±1.2 ^c	18.8±1.6 ^a	11.8±1.1	11.5±1.1	17.0±1.8 ^c	0.0001	0.0001		.0013
Mineral apposition rate (µm/d)	1.07±0.06	0.89±0.09	1.05±0.2	0.81±0.7	1.04±0.03	0.81±0.05	0.99±0.10	0.92±0.08				0.0086 ^{a*}
Bone formation rate (µm ³ /µm ² /yr)	20.7±2.0 ^{a*}	11.9±2.0	20.2±3.4	25.6±5.0 ^c	72.6±7.3 ^a	35.6±4.4	43.2±6.4	63.3±8.2 ^c	0.0001	0.0001		0.0599

Results are mean±SEM of 14-19 animals.

*OVX, ovariectomy effect; TRT, treatment effect; OVX x TRT, ovariectomy by treatment interaction

^aBasal different from vehicle, P<0.05, ^{a*}P<0.1

^bLow dose nicotine different from vehicle, P<0.05, ^{b*}P<0.1

^cHigh dose nicotine different from vehicle, P<0.05, ^{c*}P<0.1

Table 4
Histomorphometry of Midshaft Tibia (Cortical Bone) in Aged Rats After 3 Months of Nicotine Treatment

Endpoint	Sham-operated Rats				Ovx Rats				ANOVA Main Effects*			
	Low Dose		High Dose		Low Dose		High Dose		OVX P<	TRT P<	OVX x TRT P<	
	Basal	Vehicle	Nicotine	Basal	Vehicle	Nicotine	Basal	Vehicle				
Total tissue area (mm ²)	6.3±0.1	6.4±0.1	6.3±0.1	6.3±0.1	6.2±0.2	6.4±0.2	6.3±0.1	6.3±0.1				
Cortical area (mm ²)	4.2±0.1	4.3±0.1	4.2±0.1	4.0±0.1	4.0±0.1	4.0±0.1	4.0±0.1	3.9±0.1	0.0083			
Marrow area (mm ²)	2.1±0.1	2.1±0.1	2.2±0.1	2.2±0.1	2.2±0.1	2.4±0.1	2.4±0.1	2.4±0.1	0.0015		0.010 ^{b*,c}	
Periosteal Envelope												
Mineralizing surface (%)	8.2±1.7	4.6±1.8	8.8±0.9 ^b	9.1±2.1 ^c	11.5±1.9	8.1±2.0	11.3±2.6	11.3±2.6	0.0004		0.0187	0.0010
Mineral apposition rate (µm/d)	0.59±0.06	0.56±0.06	0.56±0.05	0.60±0.05	0.66±0.02	0.60±0.05	0.60±0.04	0.60±0.04	0.0373		0.0423 ^a	
Bone formation rate (µm ³ /µm ² /yr)	31.7±14.6	14.2±3.9	19.4±3.0	36.9±10.8	66.9±10.4	31.5±7.8	44.4±11.4	44.4±11.4	0.0007		0.0338 ^a	
Endocortical Envelope												
Mineralizing surface (%)	9.1±1.9	8.9±1.0	11.5±1.9	10.6±1.2	18.1±1.5	17.9±2.6	23.6±2.5	23.6±2.5	0.0001		0.0030	0.0870
Mineral apposition rate (µm/d)	0.51±0.08	0.42±0.05	0.49±0.05	0.50±0.04	0.52±0.05	0.60±0.04	0.66±0.04	0.66±0.04	0.0001			
Bone formation rate (µm ³ /µm ² /yr)	15.2±3.8	14.6±2.4	18.8±3.5	21.7±2.7	26.9±4.0	37.9±4.2	60.9±9.6	60.9±9.6	0.0001		0.0035	

Results are mean±SEM of 14-19 animals.

*OVX, ovariectomy effect; TRT, treatment effect; OVX x TRT, ovariectomy by treatment interaction

^aBasal different from vehicle, P<0.05, ^{a*} P<0.1

^bLow dose nicotine different from vehicle, P<0.05, ^{b*} P<0.1

^cHigh dose nicotine different from vehicle, P<0.05, ^{c*} P<0.1

Discussion

The objective of this investigation was to assess the effects of chronic nicotine administration on bone status and serum calcium and calcitropic hormone levels in aged, estrogen-replete (intact, sham) and estrogen-deplete (ovx) female rats. Nicotine treatment had no effect on cancellous bone mass in either the sham or the ovx animals. However, administration of nicotine at the high dose (9.0 mg/kg/day) increased mineralizing surface and bone formation rate, in both intact and ovx rats. Osteoclast surface was not affected by nicotine treatment at the cancellous envelope in these animals. This finding can be interpreted as evidence for a lack of an effect of nicotine on bone resorption. However, since resorption and formation are coupled in bone remodeling at cancellous bone surfaces, the increase in bone formation with nicotine administration without a concomitant increase in bone mass suggests that net bone resorption was also increased with nicotine treatment. In adult animals where remodeling is the predominant cellular activity, formation is often seen as an index of bone turnover²³. Earlier and later time points are needed to examine the relationship between osteoclast surface, mineralizing surface, and bone mass at the cancellous envelope and validate the hypothesis that bone resorption is increased with nicotine administration. In concordance with the lack of differences in cancellous bone mass, nicotine treatment also did not affect cortical bone mass in either intact or ovx rats. However, marrow area was greater in intact rats as well as ovx rats treated with nicotine relative to their respective vehicle-treated controls, suggesting increased endocortical bone resorption with eventual net bone loss.

In contrast to the current study, nicotine has been shown to decrease bone formation *in vitro*, inferring that the drug has an inhibitory effect on osteoblast differentiation²⁴. In our study, mineralizing surface, an index of osteoblast activation, was increased with high dose nicotine administration, whereas mineral apposition rate, an index of osteoblast activity, was unaffected by nicotine treatment. The difference in results between the current and the *in vitro* study²⁴ implies that other systemic interactions are important in modulating the effects of nicotine on bone health *in vivo*. In light of other evidence, nicotine has been shown to temporarily decrease bone formation, in association with delayed neovascularization, during bone fracture healing in rabbits²⁵⁻²⁷. Whether nicotine affects *in vivo* bone formation differently under normal conditions in which vascularization is already established than under conditions of bone injury in which vascularization is compromised remains to be determined.

In the current study, nicotine had a negative effect on 25(OH)D metabolism. Both intact and ovx rats treated with high dose nicotine had lower serum 25(OH)D levels than their respective controls. We have previously reported reduced 25(OH)D levels in some^{8,9} but not all^{10,11} of our studies which evaluated the effects of lower doses of nicotine (3.0-6.0 mg/kg/day) on bone and calcitropic hormone levels

in young and adult intact female rats. Reduced concentrations of serum 25(OH)D have also been reported in peri- and postmenopausal women smokers^{4,28,29} and elderly male smokers³⁰. Our results suggest that nicotine may be the tobacco agent responsible for the reduced serum 25(OH)D levels observed in smokers. The mechanism underlying the inverse association between smoking and 25(OH)D is not clear, but altered hepatic metabolism of 25(OH)D in the presence of nicotine may play a role⁴.

While both intact and ovx rats treated with high dose nicotine had reduced serum 25(OH)D levels, the impact of nicotine on serum 1,25(OH)₂D varied with estrogen status. Intact rats treated with high dose nicotine showed a trend for decreased serum 1,25(OH)₂D whereas ovx rats treated with the high dose nicotine showed a trend for increased serum 1,25(OH)₂D in comparison to their respective controls. Our previous studies in intact rats showed either no effect⁸⁻¹¹ or increased¹¹ 1,25(OH)₂D levels with nicotine treatment. Given the spurious associations between nicotine and serum 1,25(OH)₂D and the lack of nicotine-dependent effects on serum calcium or PTH in the current study, definitive conclusions regarding the impact of nicotine on 1,25(OH)₂D metabolism cannot be made at the present time.

Because smoking is associated with decreased efficiency of calcium absorption^{29,31}, the regulatory effects of nicotine on bone may be secondary to poor calcium nutrition. The calcium intake in the rats used in this study exceeded dietary requirements. However, nicotine may have a greater effect on bone at more marginal calcium intakes and future studies need to examine the relationship between nicotine and calcium intake on bone metabolism. In addition to its effects on calcium metabolism, smoking, or specifically nicotine, could potentially affect bone mass through changes in body mass. Chronic nicotine administration is associated with reduced food consumption³², increased energy expenditure³³, and decreased weight³⁴⁻³⁶. In concordance with other studies³⁴⁻³⁶, nicotine had a negative effect on body weight gain in the current study. Both intact and ovx rats treated with nicotine weighed less than their respective vehicle-treated controls.

The nicotine doses administered (6.0 and 9.0 mg/kg/day) in the current study resulted in average serum nicotine levels (99 to 229 ng/ml) that were approximately two to six times greater than the afternoon average for humans smoking one to two packs of cigarettes per day (40 ng/ml)³⁷. Such high doses were used because treatment with lower nicotine doses (3.0 - 6.0 mg/kg day) resulted in no or inconsistent effects on bone in our previous studies of young¹¹ and adult⁸⁻¹⁰ intact female rats. Whether higher doses of nicotine than those used in the current study would produce greater effects is unknown. However, because of complications resulting in elevated morbidity and mortality, an increase in nicotine dosage above that currently used (9 mg/kg/d) is not recommended for rats older than 12 months of age. Complications with nicotine treatment at doses up to 12 mg/kg/day were not reported by Grunberg et al.,^{34,35} in young rats (3-6 months) which may be more resilient to the toxic effects of this com-

pound than the aged rats used in this study.

Despite similar doses of nicotine administered, ovx rats had lower serum nicotine levels than intact rats. The reasons for the ovx-associated differences in serum nicotine are unclear. Cotinine, the major byproduct of nicotine metabolism³⁸, was not affected by estrogen status. While the role of another sex hormone, testosterone, has been demonstrated in nicotine metabolism³⁹, additional studies need to be conducted to examine the relationship between estrogen status and the conversion of nicotine to byproducts other than cotinine as well as the renal clearance of nicotine.

Whereas smoking results in a continuous but biphasic exposure to nicotine³⁷, administration of nicotine via osmotic minipumps results in a continuous and monophasic exposure to the drug. As such, the pattern of exposure to nicotine in the rat model used did not mimic the pattern observed in smokers. Inhalation of nicotine vapor 20 hours a day for 5 days a week for 2 years had no effect on bone mass in intact female Sprague Dawley rats¹⁷. However, administration of nicotine in drinking water for two months resulted in decreased bone mass in mice⁷. Whether the differences in results between studies using rats versus mice as a model are due to differences in patterns of nicotine exposure or species-specific differences in response to nicotine treatment remains to be determined.

The current study used retired breeder rats instead of nulliparous female rats as the experimental model. Retired breeder rats tend to be osteopenic relative to nulliparous females due to initiation of breeding prior to achievement of peak bone mass and the stress associated with multiple lactations^{40,41}. Although the cancellous bone volume was low (5-7%) in the intact rats at the start of nicotine treatment, the response of cancellous bone to ovariectomy in these retired breeder rats was similar to that reported for nulliparous females. As expected⁴², our rats lost cancellous bone following ovariectomy and the bone loss was associated with increased bone turnover at 3 and 6 months postovariectomy. In addition, as in nulliparous female rats^{43,44}, ovariectomy in the retired breeders used in the current study resulted in increased marrow area and increased endocortical and periosteal bone formation rates. Although cortical bone area was also decreased with ovariectomy in the current study, this is less commonly observed in younger nulliparous females as increased periosteal bone formation compensates for increased endocortical bone resorption.

In summary, continuous administration of nicotine at high doses resulted in an increase in bone formation rate in estrogen-replete (sham) as well as estrogen-deplete (ovx) rats indicating that nicotine may increase bone turnover. The magnitude of the increase was insufficient to significantly alter cancellous bone mass in either estrogen group over 3 months of treatment. Although marrow area was greater in rats treated with nicotine than in rats treated with vehicle, suggesting increased endocortical bone resorption with net bone loss, differences with nicotine treatment were not detected in cortical bone area. In conclusion, given the

results from this and our previous studies⁸⁻¹¹, nicotine does not appear to be the primary tobacco agent responsible for the decreased bone mass associated with smoking. However, nicotine may contribute to the decreased concentrations of 25(OH)D observed in smokers.

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