Nicotine Does Not Reduce Blood Flow to Healthy Bone in Rats

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Abstract

Given the increased incidence of orthopedic complications among smokers, we tested the null hypothesis that nicotine, the most vasoactive substance in cigarettes, does not reduce blood flow to long bones.

Nicotine was administered to adult rats at a rate of 2.4 or 3.6 mg/kg/d for 2 weeks to determine if nicotine has a dose-dependent effect on bone blood flow. Control rats received nicotine-free solution. After 2 weeks, the rats were anesthetized.

The microsphere technique was used to measure flow to femurs and tibias. Blood was collected to measure plasma nicotine. The lower dose established a plasma level of 14 ng/mL (SEM, 4 ng/mL); the higher dose elevated nicotine to 43 ng/mL (SEM, 11 ng/mL). Neither dose altered blood flow to tibias or femurs. A higher dose or longer treatment may be required to reduce bone blood flow. Alternatively, nicotine may not reduce blood flow to healthy bone at any dose but may delay bone healing by other mechanisms (ie, inhibiting angiogenesis and/or osteogenesis).

Considerable evidence suggests that smoking is a risk factor for fractures,[1-3] delayed union,[1] and nonunion.[4] According to Rhinelander,[5] adequate blood flow is essential for normal bone growth, vitality, and fracture repair. Therefore, reduced blood flow could contribute to these orthopedic complications.

Of the many chemicals in cigarettes and smoke, nicotine has received the most attention because it is largely responsible for cardiovascular effects.[6] Nicotine releases catecholamines from sympathetic nerves and the adrenal gland to elicit vasoconstriction in several vascular beds, including skin,[7] skeletal muscle,[8] and oral mucosa.[9] Like smoke, nicotine has been reported to damage the endothelial lining of blood vessels, reducing endothelium-dependent vasodilation and thereby increasing vascular tone.[10] Furthermore, nicotine has been reported to inhibit bone graft neovascularization[11] and enhance bone vascular responsiveness to norepinephrine.[12] Therefore, nicotine has the potential to reduce bone blood flow by several physiologic mechanisms.

The study reported here was designed to test the null hypothesis that nicotine does not decrease blood flow to long bones in a rat model.

Materials and Methods

Animal Model

The Institutional Animal Care and Use Committee at the University of Louisville approved all aspects of our research before it was to begin. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (Washington, DC: National Academy Press, 1996) and the *American Physiological Society Guiding Principles for Research Involving Animals and Human Beings* (approved July 2000). Adult male Sprague-Dawley rats were acclimated to the housing facility for 1 week and then randomly allocated to 1 of 3 experimental groups.

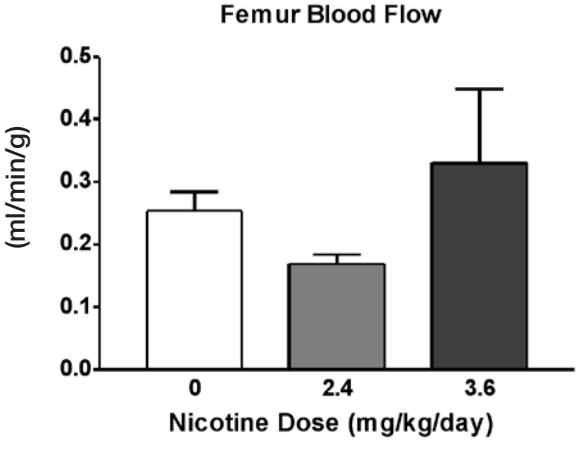

Figure 1. Femur blood flow (ml/min/g).

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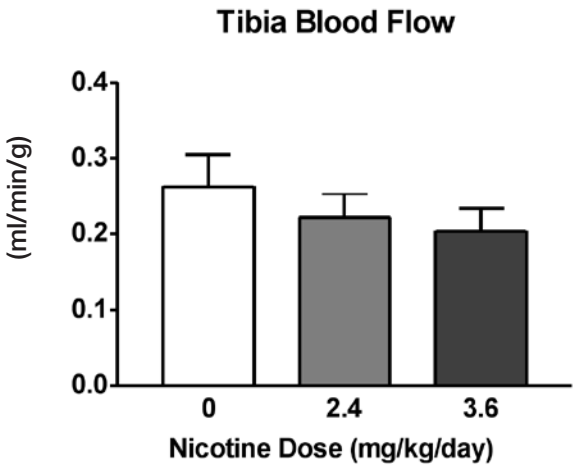

Figure 2. Tibia blood flow (ml/min/g).

Nicotine Delivery

Each rat was anesthetized with ketamine/xylazine (37.5/5 mg/kg intraperitoneal). Under aseptic conditions, a sterile osmotic minipump (Alzet model 2004, Durect Corporation, Cupertino, CA) was surgically implanted between the scapuli. The pumps implanted in control rats were filled with a nicotine-free solution of 10 mM phosphate-buffered saline (pH 7.4). Two other animal groups were implanted with pumps filled with nicotine dissolved in 10 mM phosphate-buffered saline to deliver nicotine 2.4 or 3.6 mg/kg/d for 2 weeks. The volume of solution added to each pump was considerably more than the volume to be released over the 2-week treatment period to ensure that the nicotine level was sustained. The skin incision was closed with sterile wound clips. After recovering from anesthesia, each rat was returned to the animal room. The wound clips were removed 3 to 4 days later. Throughout the 2-week period, the rats had free access to food (Test Diet Rodent 5001) and water.

Measurement of Bone Blood Flow

On the last day of the experiment, each rat was anesthetized with thiobutabarbital sodium 100 mg/kg intraperitoneal. The trachea was intubated to ensure a patent airway. A heating pad was used to maintain body temperature between 36°C and 37°C. The classical microsphere technique was used to measure blood flow to both tibias and femurs.[13-16] Flow to both kidneys was measured to assess the bilateral distribution of the microspheres. Arterial blood pressure was measured from the right carotid artery, and then the catheter was advanced to the aortic arch for microsphere infusion. The tail artery was cannulated and served as the "phantom limb." The tail was warmed with a heating pad to ensure that the artery was dilated.

After the surgical procedures were complete, the rat was allowed to stabilize for 30 minutes. Before infusion of approximately 1.5 million gold-labeled, nonradioactive microspheres (suspended in saline containing 0.01% Tween 80 and 0.01% Thimerosal), the vial was sonicated for 2 minutes and then vortexed to minimize aggregation. Fifteen seconds before microsphere infusion, a withdrawal pump was used to withdraw blood from the tail artery into a 3-mL syringe at a rate of 0.5 mL/min. The precise withdrawal rate was determined by weighing the syringe before and after blood collection and by using a digital stopwatch to measure total duration of blood withdrawal. If the withdrawal rate differed more than 10% from the expected rate of 0.5 mL/min, the experiment was excluded.

Before the rat was euthanized, a heparinized blood sample was collected and then frozen at –70°C for subsequent quantitation of plasma nicotine concentration. After the rat was euthanized, its chest was opened to confirm the location of the catheter in the aortic arch. The bones and kidneys were excised, cleaned of soft tissue, and then thoroughly rinsed with SanSaline, a physiologic solution containing a low concentration of sodium and chloride to increase the sensitivity of the assay used to quantitate the number of microspheres in each tissue. The wet weight of each tissue was determined, and then the tissues were dried overnight at 60°C, reweighed, and shipped to BioPal, Inc. for quantitation (by neutron activation) of the number of microspheres in each tissue.[17]

Tissue blood flow was calculated from the number of microspheres in the phantom limb blood sample and the precise blood withdrawal rate from the tail artery according to the formula, which is presented in the Appendix. The concentration of nicotine in the plasma of control and nicotine-treated rats on day 14 was determined by GC/mass spectrometry as described previously.[18] After each sample was thawed, 20 ng of 3',4',5'-$^{13}C_3$-(±)nicotine and 100 ng of methyl-d_3-cotinine (both from Cambridge Isotopes, Andover, Mass) were added to a 300-µL sample of the experimental plasma or standard samples containing known amounts of nicotine. Nicotine was extracted by a sequential alkaline extraction into toluene:butanol (70:30), acidic extraction into water, and a concentrated alkaline extraction into toluene:butanol (90:10). Two microliters were injected into an HP 5890 GC equipped with a DB-17 column using an HP 7673 autosampler. With oven temperature increasing from 80°C to 280°C at 25°C/min, the retention time was 4.4 minutes for nicotine. Sample detection was with an HP 5971 mass spectrometer using selected ion monitoring of m/z = 84 amu for nicotine quantified by peak-height ratio compared with the internal standards. Body weight (pretreatment, posttreatment), mean arterial blood pressure, tissue blood flow rate, and plasma nicotine levels were calculated as means and standard errors of the mean for each of the 3 experimental groups. One-way analysis of variance was used to compare the results. $P<.05$ was considered statistically significant.

Results

At the start of the experiment, the rats in all 3 groups were of comparable body weight (Table). Over the 2-week treatment period, the rats treated with nicotine-free phosphate-buffered saline or with the lower dose of nicotine gained significant weight. However, rats treated with the higher dose of

Table. Baseline Values for the 3 Experimental Rat Groups

Treatment Group (n[a])	Body Weight		Arterial Blood Pressure (mm Hg)	Plasma Nicotine Concentration (ng/mL)	Tail Artery Withdrawal Rate (mL/min)
	g	Gain (g)			
PBS (9)	260±10	67±12	119±5	0.72±0.4	0.51±0.01
2.4 mg/kg/d (6)	270±10	59±6	122±4	14±4	0.52±0.02
3.6 mg/kg/d (6)	290±3	–5±9	126±11	43±11	0.50±0.02

Abbreviation: PBS, phosphate-buffered saline.
Data are means ± standard errors of the mean.
[a]Number of rats per group.

nicotine did not gain weight (Table). Arterial blood pressure, measured on the last day of the experiment, did not differ between the 3 groups (Table). Plasma nicotine levels were elevated in a dose-dependent manner (Table). Tail artery blood withdrawal rates during the microsphere experiments were not significantly different from the expected rate of 0.05 mL/min (Table). Baseline blood flow to the femurs of control rats was 0.25±0.03 mL/min/g (Figure 1). Basal blood flow to the tibias was 0.26±0.04 mL/min/g (Figure 2). Renal blood flow was 3.5±0.3 mL/min/g. Treatment with the lower or higher dose of nicotine did not alter blood flow to the tibias, femurs, or kidneys.

Discussion

In a previous study, we demonstrated that endogenous norepinephrine participates in the regulation of blood flow to the intact rat tibia by exerting a significant constrictor effect on the vessels that supply blood to the bone.[19] Arterial blood pressure and left tibia blood flow (measured with laser Doppler flowmetry) were measured before and after administering inhibitors of α-adrenergic receptors. We reasoned that a substantial decrease in bone vascular resistance after blockade of α-adrenergic receptors would indicate that endogenous norepinephrine exerts a significant constrictor effect on the vessels that supply blood to the tibia.

First, we identified the dose of phentolamine or phenoxybenzamine that blocked the constrictor effect of exogenous norepinephrine on tibial blood flow by at least 50%. That same dose was administered to a separate group of anesthetized rats not previously treated with exogenous norepinephrine. When the antagonist was slowly infused into the left hindlimb, bone vascular resistance decreased significantly. We concluded that endogenous norepinephrine has a role in regulating basal blood flow to the rat tibia by exerting a tonic constrictor effect. Using the same approach, we determined that endothelin, a very potent vasoconstrictor peptide, does not regulate blood flow to the rat tibia, as endothelin antagonist BQ123 had no effect on bone vascular resistance.[20] We conclude that not all endogenous constrictor agonists participate in bone blood flow regulation.

Given evidence that nicotine amplifies the constrictor effect of norepinephrine in other vascular beds,[7-9] we investigated whether nicotine augments the bone vascular responsiveness to exogenous norepinephrine.[12] Nicotine was delivered subcutaneously to adult male rats via osmotic minipumps at a dose of 1.7 mg/kg/d for 2 weeks. At the end of the treatment period, the rats were anesthetized, and bone blood flow and arterial blood pressure were measured. Responsiveness of the bone vasculature to norepinephrine and vasopressin was assessed in separate groups of animals by administering 5 increasing doses of each agonist into the left hindlimb. Nicotine significantly enhanced bone vascular response to norepinephrine but not to vasopressin. Therefore, nicotine appear not to augment the bone vascular response to all endogenous constrictor agonists.

As nicotine enhanced the constrictor effect of norepinephrine on the bone vasculature, we wondered whether nicotine, acting through multiple physiologic mechanisms, would reduce blood flow to long bones. Nicotine was administered for 2 weeks, as significant enhancement of bone vascular responsiveness to norepinephrine occurred within that same period in our previous study.[12] Two doses were administered to determine if nicotine has a dose-dependent effect on bone blood flow. At the end of the treatment period, microsphere experiments were performed to quantitate blood flow to the femurs and tibias. Flow to both kidneys was measured to determine if the microspheres were distributed bilaterally. Plasma level of nicotine in each rat on the last day of the experiment confirmed that the osmotic pumps released nicotine throughout the 2-week treatment period and that 2 distinct levels of nicotine were achieved (Table).

Rats that received the lower dose of nicotine gained as much weight as rats that received the nicotine-free solution, but rats that received the higher dose of nicotine did not gain weight (Table). An inverse relationship between body weight gain and nicotine dose is well documented in rats.[21] Baseline blood flow to the femurs and tibias of control rats (Table) was well within the range reported by others using the same microsphere approach.[13-16] Although the higher nicotine dose significantly suppressed weight gain, it had no effect on bone blood flow.

There may be several reasons why nicotine had no effect on bone blood flow in this study. The dose and/or duration of nicotine treatment may not have been sufficient to alter the physiologic mechanisms that regulate bone blood flow. Many humans smoke for half to two-thirds of their lives (40-50 years). A comparable period in the life of a rat

(8-12 months) might be required to significantly reduce bone blood flow. Second, the nicotine doses may not have been sufficient to alter bone vascular function. Alternatively, nicotine may not alter blood flow to healthy bone at any dose but may have effects on bone vasculature after bone injury. According to Trueta,[22] blood flow decreases substantially within the first few weeks after a bone fracture. Afterward, flow increases and remains elevated for several weeks. The increase in flow is thought to be crucial for normal bone healing. Daftari and colleagues[11] found that nicotine inhibits angiogenesis, potentially delaying bone healing. Furthermore, nicotine has been reported to suppress proliferation of osteoblast-like cells[23] and to inhibit fibroblast migration.[24] Both actions could delay bone repair. A final consideration is that toxic chemicals in cigarettes and cigarette smoke, other than nicotine, may account for the orthopedic complications among smokers.[25]

Given our study results, we conclude that nicotine, administered for 14 days at a lower or higher dose, does not affect blood flow to the tibia or femur in a rat model.

Authors' Disclosure Statement

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Appendix

Tissue blood flow was calculated from the number of microspheres in the phantom limb blood sample and the precise blood withdrawal rate from the tail artery according to the formula:

$$\text{Tissue Blood Flow} = \frac{\text{Tissue Microsphere Count} \times \text{Phantom Limb Blood Flow Rate}}{\text{Phantom Limb Microsphere Count}}$$