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Bone Biomechanical Property Deterioration Due to Tobacco Smoke Exposure

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Abstract. Tobacco smoking has been implicated in the development of osteoporosis and early onset of menopause in women smokers. We measured various biomechanical properties of femurs and tibiae obtained from smoke-exposed and control mice to determine cigarette smoke influences on bone mass, structure, and strength. Growing female C57BL mice were exposed to sidestream cigarette smoke in a whole-body exposure chamber, set at 30 ± 2 mg smoke particulates/m^3 for 4 hours/day and 5 days/week for 12 consecutive weeks. Elevated levels of urinary cotinine and pulmonary ethoxyresorufin deethylase activity in smoke-exposed mice confirmed their effective exposure to cigarette smoke. There were no differences in body weight and physical size (length, medial-lateral and anterior-posterior widths, midshaft cortical area and thickness) of femurs and tibiae between smoke-exposed and control mice. The femoral mid-shaft yield load, stiffness, yield stress, and modulus were, respectively 8%, 13%, 10%, and 14% lower ($P < 0.05$) in smoke-exposed compared to control mice. The ultimate load and stress in mid-shaft femurs showed decreasing trends ($P < 0.1$) in smoke-exposed mice. In the femoral neck, the ultimate load and stiffness were 9% and 12% lower ($P < 0.05$) in smoke-exposed mice, respectively. Further, the ash-to-dry bone weight ratio was smaller (~6%, $P < 0.05$), and micro-computed tomographic scanning of distal femoral bone volume/total volume (%) and trabecular thickness showed decreasing trends in smoke-exposed mice compared to the control group. We conclude that exposure to tobacco smoke deteriorates some of the biomechanical properties of bone in growing female mice.

Key words: Tobacco — Smoking — Cigarette — Bone mineral content — Bone mineral density — Bone strength — Mouse

Tobacco (cigarette) smoking is a major health risk that increases an individual's health-care costs and decreases life expectancy [1–6]. Smoking has been strongly implicated in cancers of various organ sites (e.g., lung, bladder, pancreas, etc.) [7–9] and in various cardiovascular and respiratory diseases [10–12]. In addition, the use of tobacco is associated with low bone mass and increased fragility fracture risk [11, 13–23]. Several studies suggest that cigarette smoking exerts antiestrogenic effects in females, resulting in an increased incidence of early menopause and osteoporosis (bone fragility) in smokers [24–30]. An earlier onset of menopause by 1–2 years and a dose response has been reported in women smokers who smoke more than 10 cigarettes/day [31, 32]. On average, women are at greater risk of bone loss leading to skeletal fractures compared to men [33, 34], and smoking further increases their skeletal fragility risk [16, 23, 34, 35]. Based on a study of twin pairs discordant for cigarette use, Hopper and Seeman [14] calculated that, by the age of menopause, women who smoke one pack of cigarettes/day throughout life will have 5–8% less bone than nonsmokers. Others have observed 5–10% less bone and reduced protective effects of nutritional calcium in postmenopausal smokers than nonsmokers [15–17, 35–42]. These studies provide evidence that smoking-related bone fragility is a critical problem in women and warrants examination of the relationship between cigarette smoking and bone mechanical properties.

Nicotine is the principal pharmacologically active chemical in tobacco [43] and has been extensively studied in experimental models. However, its effects on bone strength in a noninjury animal model have been poorly defined [44–49]. Our past studies in intact (young to adult) and estrogen-replete rats given various doses of nicotine (3–9 mg/kg daily) have found some effects on the biomechanical properties of bone [44–46, 48, 49]. Although nicotine has been shown to compromise mechanical strength properties of bone during fracture healing [50], its effects on bone biomechanical properties are mixed [44–46, 48, 49]. In studies where osmotic pumps were used to continuously deliver nicotine (doses of 3.0 and 4.5 mg/kg daily) in rats, the data suggest no

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difference [44–46] to decreased bone strength values [48, 49] in some biomechanical properties of nicotine-treated rats compared to controls. In addition, high-dose nicotine (6 and 9 mg/kg daily) in an animal model of postmenopausal bone loss suggested a marginal effect on a few biomechanical properties of bone [48, 49]. Even with a sufficiently high intake of nicotine (6–9 mg/kg daily), the expected compromise in bone mass and strength was small [48] in the estrogen-replete (intact) and estrogen-depleted (ovariectomized) rats, a well-established animal model of postmenopausal bone loss [48, 49]. However, tobacco smoke exposure was found to be more detrimental to a bone and implant interface (in terms of bone/implant contact area) than nicotine treatment alone in the tibiae of adult Wistar rats [51]. These data suggested that tobacco smoke constituents other than nicotine might be responsible for the compromised biomechanical properties in the skeleton of smokers.

Recently, the research focus has shifted toward determining the role of genetics in skeletal health of murine models [52–57]. Therefore, our interest was in determining the effect of environmental insults, such as exposure to tobacco smoke, on bone properties in the mouse model. The main aim of the present study was to evaluate the effects of whole tobacco smoke exposure on bone mass, structure, and strength in growing virgin female mice.

Materials and Methods

Eight-week-old female C57BL (apoE$^{-/-}$) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained as described previously [58]. After acclimatization for 2 weeks, animals were randomly divided into two groups: sham-exposed (n = 20), maintained as a control group in filtered ambient air, and smoke-exposed (n = 32), exposed to sidestream cigarette smoke as described earlier [58]. Mice were housed four to a cage and received water and a "Western type" diet (Teklad 88137, Harlan Teklad, Madison, WI, USA) *ad libitum* for the duration of the study.

All inhalation exposures to smoke were carried out in a whole-body Hinners-type stainless steel/glass chamber [58]. Briefly, animals were exposed to sidestream cigarette smoke in a chamber maintained at a smoke particulate concentration of about 30 mg/m^3 for 4 hours/day, 5 days/week for a total of 12 weeks. Inhalation of smoke by the animals was monitored by measuring urinary cotinine during exposures and the induction of ethoxyresorufin deethylase (EROD) activity in lung microsomes. Urinary cotinine was measured by an enzyme-linked immunosorbent assay and EROD activity, by spectrofluorimetry, as described previously [58].

At the time of necropsy, femurs and tibiae were excised, cleaned of soft tissue, and stored in saline at –20°C for subsequent bone biomechanical strength, mass, and structure measurements.

Bone Strength

Femurs were tested by three-point bending with force applied to the anterior surface. The loading was such that the anterior surface of the femur (mid-shaft) was in compression and the posterior surface was in tension. After strength testing at mid-shaft, the femoral neck was tested by bending. Force was applied to the femoral head in a direction parallel to the shaft length [48, 59, 60]. All biomechanical tests were conducted at room temperature and at a rate of 3 mm/minute using a servocontrolled mechanical testing machine (Instron 5543, Canton, MA). Load-deformation curves were plotted and analyzed for structural strength parameters such as ultimate load, yield load, and stiffness [61]. *Ultimate load* was defined as the maximum load that a specimen (maximum height of the curve) takes before fracturing. *Yield load* is the load at which permanent damage/deformation is incurred in the specimen. The yield load was estimated to be the intersection point of the load displacement curve and a line parallel to the linear portion of the load-displacement curve but offset by 0.2% of the initial specimen length [61, 62]. *Stiffness* is the slope of the linear portion of the load-displacement curve.

Two transverse femoral cross-sections adjacent to the fracture site at the mid-shaft were first traced at ×20 on tracing papers using a profile projector (V-10; Nikon, Tokyo, Japan) and then digitized on a VaxStation 2000 computer (Digital Equipment Corp., Maynard, MA, USA) using a digitizing tablet [60]. Average radii, second moment of area/inertia about the medial-lateral axis, and cross-sectional cortical areas were determined using the program SECTION, developed at the Creighton University Osteoporosis Research Center Biomechanics Laboratory. Femoral mid-shaft apparent material strength properties (ultimate stress, yield stress, flexural modulus) were calculated as load/cross-sectional area [61, 63].

Bone Mass Measurements

After measuring length and mid-shaft widths (in the medial-lateral and anterior-posterior directions), tibial bone specimens were used to measure bone percent ash content (bone dry weight/ash weight) using standard bone ash techniques (600°C oven) [64, 65]. We used a digital caliper (Mitutoyo, Kawasaki, Japan) for the bone length and width measurements. The anatomical site was 2.9 mm proximal to the tibiofibular junction in tibiae.

Micro-Computed Tomographic Analysis

Using a micro-computed tomography (μCT) device (μ-CT-20; Scanco Medical, Bassersdorf, Switzerland), distal femurs were scanned to determine cancellous/trabecular structural properties in terms of bone volume/total volume (BV/TV), trabecular thickness, spacing, and number. The length of the distal femur scans was 4 mm from the distal end. The distal femur scans were performed at 9 μ resolution, with an integration time of 80 milliseconds, and using standard techniques as described previously [66, 67].

Statistical Analysis

Student's t-test (SPSS, Chicago, IL; v12.0) was used to find differences in all the measured variables between smoke-exposed and control groups. The level of statistical significance was set at $P \leq 0.05$. Data are reported as mean ± standard error of the mean (SEM). The pooled standard deviation (SD) was calculated to estimate the common SD for all measured variables.

Results

Biochemical and Physical Measurements

Animals appeared generally healthy, with no loss due to smoke exposure. No significant differences were observed in the body weights of control (22.82 ± 1.54)

Table 1. Physical measurements (mean ± SEM)

	Control (sham)	Smoke-exposed
Femur		
Length (mm)	15.4 ± 0.05	15.1 ± 0.05
Medial-lateral width (mm)	1.6 ± 0.03	1.6 ± 0.01
Anterior-posterior width (mm)	1.0 ± 0.03	1.0 ± 0.01
Second moment of area (mm^4)	0.123 ± 0.006	0.120 ± 0.004
Mid-shaft cortical area (mm^2)	0.790 ± 0.03	0.785 ± 0.02
Midshaft cortical thickness (mm)	0.18 ± 0.01	0.18 ± 0.01
Tibia		
Length (mm)	17.1 ± 0.1	17.5 ± 0.1
Medial-lateral width (mm)[a]	1.5 ± 0.03	1.6 ± 0.01
Anterior-posterior width (mm)[a]	1.1 ± 0.02	1.1 ± 0.01

[a] Tibial width (both medial-lateral and anterior-posterior) was measured at 2.9 mm proximal to the tibiofibular junction

and exposed (22.24 ± 1.53) groups after 12 weeks of smoke exposure. Elevated levels of urinary cotinine and pulmonary EROD activity confirmed the exposure of mice to smoke. Urinary cotinine levels in smoke-exposed animals ranged 2.46–3.54 μg/mg creatinine in comparison to negligible levels in the control group. Similarly, EROD activity (mean ± SEM) of lung microsomes increased severalfold in smoke-exposed [23.5 ± 4 pmole/(min·mg)] compared to control [1.8 ± 0.2 pmole/(min·mg)] mice.

There were no differences in the femoral and tibial shaft physical measurements (length, width, second moment of area/inertia, cortical area, cortical thickness, etc.) between smoke-exposed and control mice (Table 1).

Bone Strength

Both the structural and apparent material strength parameters of mid-shaft femurs either were significantly lower ($P < 0.05$) (Figs. 1 and 2) or showed some deterioration ($P < 0.1$) in the smoke-exposed group (Table 2). The femoral mid-shaft (three-point bending test) yield load, stiffness, yield stress, and modulus were, respectively, 8% (SD = 0.61), 13% (SD = 0.90), 10% (SD = 0.3), and 14% (SD = 0.58) lower ($P < 0.05$) in smoke-exposed compared to control mice (Table 2, Figs. 1 and 2). The ultimate load and stress in mid-shaft femurs showed decreasing trends ($P < 0.1$) in smoke-exposed mice. In the femoral neck, ultimate load and stiffness were 9% (SD = 0.63) and 12% (SD = 0.43) lower ($P < 0.05$) in smoke-exposed mice, respectively (Table 2, Fig. 3).

Bone Mass/Ash

The effects of treatment on femoral bone mass/ash weight are presented in Table 3. While no differences were noted in dry bone and ash weights, the ash-to-dry bone weight ratio was smaller (~6%, $P < 0.05$) in smoke-exposed mice compared to the control group (Table 3).

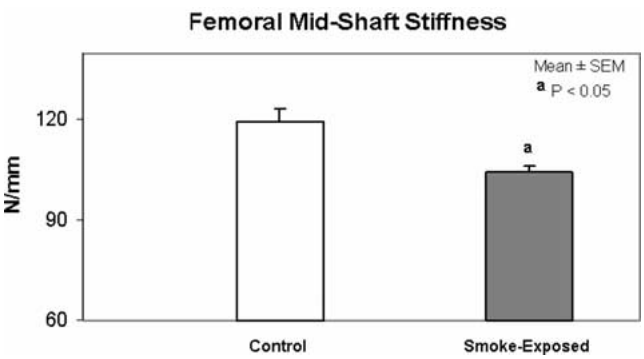

Fig. 1. Femoral mid-shaft stiffness in three-point bending test. Open and shaded bars represent bone stiffness in control and smoke-exposed mice, respectively. Mid-shaft femoral stiffness in smoke-exposed mice was significantly lower ($P < 0.05$) compared to controls.

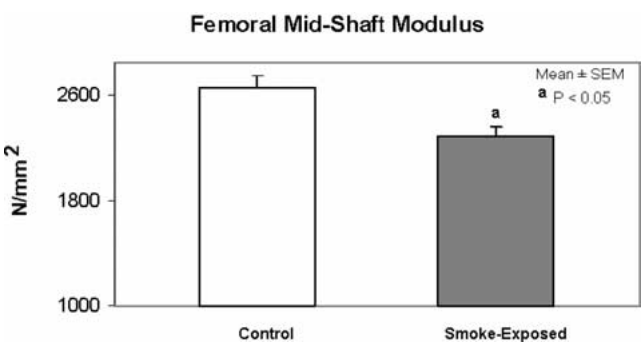

Fig. 2. Femoral mid-shaft modulus in three-point bending test. Open and shaded bars represent bone modulus in control and smoke-exposed mice, respectively. Mid-shaft femoral modulus in smoke-exposed mice was significantly lower ($P < 0.05$) compared to controls.

μCT Analysis

None of the μCT measured parameters (Table 4) was significantly different between the smoke-exposed and control groups. However, while the ratio BV/TV and trabecular thickness in the distal femur showed

Table 2. Biomechanical properties (mean ± SEM)

	Control (sham)	Smoke-exposed
Femoral shaft		
Ultimate load (N)	18.2 ± 0.4	17.3 ± 0.2[b]
Yield load (N)	15.3 ± 0.4	14.0 ± 0.2[a]
Ultimate stress (N/mm^2)	118 ± 3	111 ± 2[b]
Yield stress (N/mm^2)	100 ± 4	90 ± 3[a]
Femoral neck		
Ultimate load (N)	17.4 ± 0.4	15.9 ± 0.3[a]
Yield load (N)	8.8 ± 0.6	9.0 ± 0.3

Different from control: [a]$P < 0.05$, [b]$P < 0.1$
N, Newton

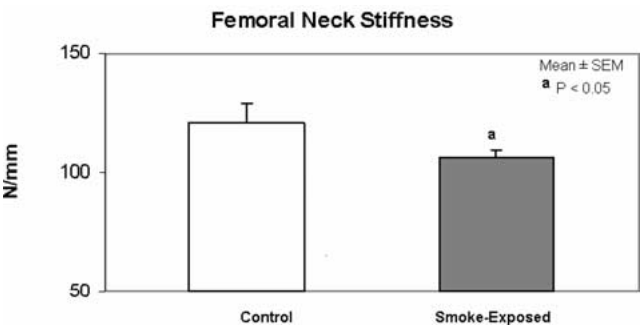

Fig. 3. Femoral neck stiffness in three-point bending test. Open and shaded bars represent bone stiffness in control and smoke-exposed mice, respectively. Femoral neck stiffness in smoke-exposed mice was significantly lower ($P < 0.05$) compared to controls.

Table 3. Tibial bone mass (mean ± SEM)

	Control (sham)	Smoke-exposed
Dry weight (mg)	27.9 ± 0.7	29.5 ± 0.6
Ash weight (mg)	11.2 ± 0.3	11.3 ± 0.3
Ash/dry weight ratio	0.403 ± 0.005	0.380 ± 0.008[a]

Different from control: [a]$P < 0.05$

decreasing trends ($P < 0.1$) in the smoke-exposed group, there were no differences in trabecular number and spacing between the smoke-exposed and control mice (Table 4).

Table 4. Distal femur trabecular bone structure (mean ± SEM)

	Control (sham)	Smoke-exposed
Trabecular BV/TV (%)	0.458 ± 0.005	0.438 ± 0.001[b]
Trabecular number (mm)	17.3 ± 0.5	18.3 ± 0.4
Trabecular thickness (mm)	0.067 ± 0.002	0.065 ± 0.001[b]
Trabecular spacing (mm)	0.065 ± 0.003	0.061 ± 0.002

Different from control: [b]$P < 0.1$

Discussion

This study evaluated the effects of sidestream cigarette smoke exposure (a total of 240 hours over 12 weeks) on bone biomechanical properties, bone mass, and structure in growing female mice, an animal model that has been used for the study of genetic influences on bone biomechanical properties [52–56]. The growing mouse model may allow us to examine skeletal effects in teenagers in which smoking is quite prevalent. There was a significant decrease in most of the structural strength (yield load, stiffness) and apparent material (yield stress, flexural modulus) properties of the femoral mid-shaft, while only the structural strength (ultimate and yield load) properties in the femoral neck were lower for smoke-exposed compared to control mice. In addition, measurements of tibial bone mass (ash weight/dry weight ratio) and distal femur structural properties (μCT) suggest a lower tibial bone ash weight ratio and declining trends of %BV/TV in the smoke-exposed group. These results clearly suggest deleterious effects of smoke exposure on biomechanical properties of bone in the intact (estrogen-replete) mouse model. We recognize that smoking and the depletion of estrogen in postmenopausal women create significantly lower bone mass and increase the risk of bone fragility fractures [25, 26, 29, 68]. The intent of the present study was to establish baseline data on the effect of smoking on bone properties; therefore, the estrogen status of the mice was not determined. Future studies to examine estrogen and its metabolites in intact and ovariectomized animals will be needed to examine the mechanisms of the smoke effect. Thus, further tobacco smoking studies on bone fragility are warranted. This baseline study will allow future studies to test whether compromised bone biomechanical properties are due to smoking-related defects in collagen composition, cross-linking, or modeling/remodeling of periosteal/endosteal surfaces [69, 70].

The hypothesis was that tobacco smoke exposure may cause bone-structure and apparent material strength property changes. The bone apparent material strength properties may be influenced by changes in collagen fiber composition and cross-linking. It has been shown that any blocking of the cross-linking in bone causes poor mineralization, which may lead to compromised bone-strength properties. In human tobacco smokers, the marker bone turnover N-terminal collagen cross-links (NTx) shows increased levels [20] compared to nonsmokers. In addition, these data [20] suggest that any smoking-related increased bone turnover may cause skeletal fragility.

The exposure of mice to tobacco smoke under our experimental conditions significantly raised urinary cotinine levels (2.46–3.53 μg cotinine/mg creatinine) above those in the control group. These urinary cotinine levels suggest that the smoke exposure of these mice was equivalent to the exposure reported for human smokers who smoke about 10–15 cigarettes/day [71–73]. Unlike

nicotine alone [44–47, 74], the tobacco (cigarette) smoke exposure did compromise some of the biomechanical properties in this study. Previous studies of nicotine administration in rats (1.5- to 4.5-fold compared to human chronic smokers) [74] showed no effect on biomechanical properties at low doses (3–4.5 mg/kg daily) [44–47]. Further, despite the range of doses (4.5–9 mg/kg daily) used, only the higher nicotine dose (9 mg/kg daily) had limited harmful effects on vertebral bone mineral content (BMC) [49], femoral ultimate load, yield load, and yield stress [48, 49] in adult female intact and ovariectomized rats. These data [48, 49] suggest that nicotine has a minimal effect on the biomechanical properties (structural and apparent material strength) in both ovariectomized and sham-treated rats even at a high dose (9 mg/kg daily) of nicotine [48]. Similar to high doses, the lower nicotine doses (3–4.5 mg/kg daily) in growing to adult female rats that produced serum concentrations similar to those in smokers also had no effect on the biomechanical properties of bone [44–47]. However, unlike the nicotine treatment studies, the present study clearly suggests some deleterious effects of whole tobacco smoke exposure in mice on some bone biomechanical properties. While some variables (Tables 2–4, Figs. 1–3) showed significant biomechanical strength declines in the smoke-exposed group, the other variables (Tables 2–4) did not reach statistical significance; therefore, they should not be considered different between smoke-exposed and control mice. The few biomechanical properties that were significantly different (Tables 2–4, Figs. 1–3) allow us to conclude that tobacco smoking has adverse skeletal effects. However, it is likely that longer exposure to smoke may further magnify these skeletal defects in the relatively healthy bones of growing mice. In addition, exposure to tobacco smoke may evoke a differential response in rats and mice and should be considered when making a direct comparison across different species.

In studies involving determination of the harmful effects of pure tobacco smoke constituents such as nicotine and polycyclic aromatic hydrocarbons (PAHs) on biomechanical properties of bone, the route of administration may also influence the outcome. For instance, consistent with our earlier rat studies [44–46], nicotine vapor administration via inhalation (which caused plasma nicotine levels to increase to the levels of heavy smokers) did not affect femoral strength in Sprague-Dawley rats even after a 2-year exposure [47]. However, administration of nicotine in drinking water for 2 months decreased BMC and bone mineral density (BMD) in mice [75]. Similarly, studies of other smoke constituents have shown that administration of benzo[*a*]pyrene (BaP) and 7,12-dimethylbenz[*a*]anthracene (DMBA) via the subcutaneous route (250 μg/kg of BaP/DMBA weekly for 15 weeks) causes a significant decline in BMD and bone strength of adult Sprague-Dawley ovariectomized rats [76].

The negative effects of tobacco smoke and its constituents such as nicotine and PAHs on bone may result from slow healing, poor bone and implant interface, increased resorption, slower growth and lengthening, and increased bone fragility in animal models [51, 77–79]. The harmful effects of whole smoke exposure on bone in the present study are evident from the tibial bone ash ratio (Table 3) and the structural and apparent material properties of femurs (Table 2, Figs. 1–3). Both yield load (structural strength) and yield stress (apparent material strength) properties are significantly decreased in the smoke-exposed compared to control mice (Table 2), suggesting that smoking changes the material properties of bone such that the force threshold for accumulation of permanent damage declines significantly. The lower yield strength properties may result from the deterioration of collagen fiber essential for the health of bone tissue. It is well documented that smoke exposure modulates collagen fiber in lungs [80, 81]. It is, therefore, likely that it also adversely affects collagen in bones, which in turn influences their yield strength properties. Higher levels of NTx in smokers, suggesting greater bone turnover, have been reported [20]. These observations suggest that increased bone turnover by cigarette smoking may play a role in skeletal fragility and the reduction in the yield strength properties of bones.

Unlike yield load and stress, the femoral mid-shaft ultimate load and stress (Table 3) did not decrease significantly in the smoking group, suggesting that whole bone post-yield maximum strength is not as sensitive to changes in the bone properties in mice exposed to 12 weeks of cigarette smoking.

BMC and BMD in women smokers are lower, putting them at a greater risk of skeletal fractures compared to nonsmokers [16, 23, 26, 34, 38, 39]. Lower bone mass in smokers may be due to thinning of cortical bone, lower trabecular numbers, trabecular thickness, and greater trabecular spacing in cancellous bone. Although no cortical size differences (Table 1) were noted, the bone mass and declining trends (Table 3, 4) of trabecular/cancellous bone architecture in this animal study is consistent with the poor bone mass conditions observed in humans [24–30].

Lower ash-to-dry weight ratios of smoke-exposed mice bones suggest relatively lower BMC of the tibial specimens. Assuming that similar trends also exist in the femoral bone site, it could be speculated that lower BMC is responsible for the lower structural and apparent material strength properties measured in smoke-exposed mice (Table 2, Figs. 1–3). Although %BV/TV and trabecular thickness in the distal femurs showed trends of being smaller in the smoke-exposed group, trabecular number and spacing were not different (Table 4). This suggests that the overall trabecular bone in the smoke-exposed group is weaker even though μCT

failed to detect defects in other parameters of bone structure. In addition, despite similar bone size at the mid-shaft femur (Table 1), either significantly lower or decreasing femoral structural strength suggests that cigarette/tobacco smoke negatively affects the apparent material properties (ultimate/yield stress, modulus; Table 3, Figs. 1–3), thus changing its intrinsic material strength properties [82]. Therefore, future experiments pertaining to tobacco smoke exposure and skeletal health should include characterization of bone intrinsic properties using techniques like nano-indentation.

Duration of cigarette smoking in humans is much longer than what has been used for animal studies. A chronic smoker smokes for an average of 18 years [83], which translates into 34% of their lifetime (average age 53 years) [84]. In animal studies (mice, 24-month life span), the human equivalent time period translates into 32 weeks of smoking. However, the 12-week duration of tobacco smoke at a concentration of 30 mg/m^3 TSP (total suspended particulates) seems to be enough to negatively influence some of the bone (both cortical and trabecular) biomechanical properties. The harmful effects or changes in bone properties may be greater at higher-level exposures to tobacco smoke than those used in the present study.

The effect of smoking in postmenopausal women is even greater. It leads to considerably lower bone mass and density [68], reflecting the negative effects of both estrogen loss and tobacco smoke. The current study did not examine the effects of smoke exposure on bone health in ovariectomized mice. The combined influences of estrogen loss and tobacco smoke on bone biomechanical properties will be deleterious and should be quantified in future studies.

In summary, this study demonstrates that tobacco smoke exposure has significant detrimental effects on bone mass and bone biomechanical properties in growing female mice.

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