Tobacco Extract but Not Nicotine Impairs the Mechanical Strength of Fracture Healing in Rats

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ABSTRACT: The influence of nicotine and tobacco extract (without nicotine) alone and in combination on and mechanical strength of closed femoral fractures in rats was investigated. One hundred four male Sprague-Dawley rats were divided into four groups receiving: nicotine, tobacco extract, tobacco extract plus nicotine, and saline. One week prior to fracture, osmotic pumps were implanted subcutaneously in all animals to administer nicotine equivalent to the serum level of nicotine observed in a smoker consuming one to two packs of cigarettes daily. An equivalent volume of saline was administered to the control animals. Tobacco extract was administered orally. A closed transverse femoral diaphysial fracture was performed, and stabilized with an intramedullary pin. The fractures were mechanically tested after 21 days of healing. Tobacco extract alone decreased the mechanical strength. Ultimate torque and torque at yield point of the tobacco extract group were decreased by 21% ($p = 0.010$) and 23% ($p = 0.056$), respectively, compared with the vehicle (saline) group, and by 20% ($p = 0.023$) and 26% ($p = 0.004$), respectively, compared with the nicotine group. No difference was found between the tobacco extract and tobacco extract plus nicotine groups. An 18% ($p = 0.013$) reduction in torque at yield point was observed in the tobacco extract plus nicotine group compared with the nicotine group. No differences in ultimate stiffness, energy absorption, and callus bone mineral content at the fracture line were found between any of the groups. Serum levels of nicotine were between 40–50 ng/mL in the group given nicotine alone and the group given tobacco extract plus nicotine (equivalent to serum levels observed in persons smoking one to two packs of cigarettes per day). © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:1472–1479, 2006

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INTRODUCTION

Fracture repair is slower in smokers compared to people who do not smoke, and the rate of nonunion is higher among smokers. This has been shown in clinical trials concerning both open/closed tibiae shaft fractures and spinal fusions.[1–5] Furthermore, these findings have been supported by a number of fracture and spinal fusion studies in rodents exposed to different levels of nicotine.[6–9] The negative impact might be explained by nicotine's inhibition of osteoblastic activity[6,10] and by its vasoconstrictive action on the microvasculature.[11–13] The vasoconstrictive effect has been observed during revascularization of cancellous bone graft during spinal fusion.[14] It might also be that nicotine attenuates a wide range of cytokines that are normally expressed during bone formation.[15]

A common feature of the animal studies above was that the experiments used only nicotine, and the nicotine was usually administered in doses causing higher serum levels than those observed in smokers consuming one pack of cigarettes daily.[11,16,17] Besides nicotine, there are about 3500 other compounds in the particulate phase of cigarette smoke, including some of the lung carcinogenic agents.[11,13,18] Which of these multiple compounds that may inflict on bone biology, still remains unknown.

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To the best of our knowledge, no experiment has been previously conducted to examine the effect of the tobacco extracts without nicotine on fracture healing. Therefore, the aim of this study was to investigate the effects of tobacco extract and nicotine, when given separately and in combination, on the mechanical strength of fracture healing in the rat. The tobacco extract was obtained from nicotine free cigarettes and administered orally. Nicotine was administered continuously in a dose that resulted in serum levels equivalent to those observed in daily smokers.[11,13,16,17,19]

MATERIALS AND METHODS

Animals and Design

The experimental protocol was approved by Institutional Animal Care and Use Committee, Minneapolis Medical Research Foundation.

One hundred four male Sprague-Dawley rats with an initial weight of 250–275 gram were randomized according to weight into four groups, receiving nicotine, tobacco extract, tobacco extract plus nicotine, and saline. The rats were housed individually and allowed food and water ad libitum, using tobacco extract as night drink in the tobacco extract and tobacco extract plus nicotine groups. The animal housing room was maintained on a 12-h light/dark cycle (the light cycle began at 07.00 h) at approximately 21°C.

Treatment was administered for 4 weeks—starting 1 week prefracture and continuing 3 weeks postfracture.

Preparation and Administration of Nicotine

Nicotine bitartrate was obtained from Sigma Chemical Co. (St. Louis, MO). Osmotic minipumps (Alzet 2ML4, Durect Cupertino, CA) were loaded with the nicotine preparation containing a concentration of 14.00 mg/mL. The 2ML4 pumps delivered 2.5 μL/h for 4 weeks, which yielded a dosage of 3.0 mg/kg/day.

The pumps were implanted subcutaneously (under intraperitoneal ketamine and xylazine anesthesia at a dose of 80–100 mg/kg and 2–3 mg/kg, respectively) in the intrascapular area using aseptic conditions. All animals received a pump containing either saline (vehicle) or the nicotine solution.

Preparation and Administration of Tobacco Extract

Tobacco extract was prepared, immediately prior to administration from nicotine-free Quest Cigarettes (Vector Tobacco Inc., Timberlake, NC), as described by Demady et al.[17] Four cigarettes were cut open, and the tobacco was ground with a mortar and pestle and placed in a 50-mL plastic tube containing 40 mL water. The tubes were mixed overnight at room temperature using a tube tipper. The contents were filtered through gauze and the liquid was centrifuged at 5000 rpm for 20 min. The supernatant was vacuum filtered using Whatman paper and then filtered again using a 0.2-μm sterile filter. The tobacco extract was diluted 1/30 before administration. The solution was administered orally for 16 h/day, with water provided for the remaining 8 h. The daily amount of tobacco extract consumed at the end of the 16-h period was recorded. The concentration of anatabine, a minor tobacco alkaloid, in the tobacco extract was measured by gas chromatography-mass spectrometry.[20]

The concentration of tobacco extract to be provided in drinking water was determined by first extracting tobacco from cigarettes containing nicotine (Quest Low Nicotine) and then calculating how much of this tobacco the rats would need to drink daily to ingest 3 mg/kg of nicotine (1.0 mL). A 1:30 dilution of the tobacco provided in place of drinking water for 16 h/day caused an approximately 3 mg/kg dose of nicotine, as the rat drank an average of 30 mL of the solution during the 16-h exposure period. The same procedure was used to produce a similar dilution (tobacco extract) from nicotine free cigarettes (Quest No Nicotine).

Closed Fracture Surgery of Left Femur

The animals were anesthetized using intraperitoneal injections of ketamine/xylazine at a dose of 100 mg/kg and 2.0 mg/kg, respectively. When the animals reached the surgical stage of general anesthesia, preparation for surgery was initiated, including shaving and disinfection of the left leg. A lateral parapatellar approach to the femoral condyle was performed by a 5-mm skin incision (without damaging the patellar ligament). The longitudinal fibers of the quadriceps mechanism were divided and the patella was dislocated medially. Internal rotation of the tibia and valgus of the knee resulted in exposing of the femoral condyle. The marrow canal was reamed with a 1.5-mm 18-gauge needle. A 1.4-mm K-wire (D. Trocar PT 9, 054 in) of 33 mm in length was introduced in a retrograde fashion. The pin was countersunk and seated in the proximal femur. Muscles and fascia were reapposed using Ethilon 4-0 suture (Ethicon) and the skin was closed in standard fashion, also using Ethilon 4-0 suture.

Immediately after K-wire placement, a closed transverse fracture in the mid-diaphyseal region of the femur was performed using a fracture apparatus designed according to the original specifications by Bonnarens and Einhorn.[21] The presence of the transversal fracture line was confirmed by palpation.

Serum Analysis

To determine nicotine and cotinine levels, blood samples were taken from a tail vein at fracture, after 2 weeks of healing, and at sacrifice. Then serum was stored at −20°C until assayed by gas chromatography (Hewlett-Packed 5890 Series II) with nitrogen-phosphorus.[22]

Mechanical Testing

Three weeks following fracture surgery, the rats were killed with pentobarbital (100 mg/kg intraperitoneally; Beauthanasia-D). X-rays of the femurs were taken to inspect fracture healing and position of the

intramedullary K-wire. Femurs were stripped of soft tissue and the K-wire was carefully removed by gentle axial pull with rotational motions, taking care not to disrupt the healing tissue. The proximal and distal ends were potted in aluminum fixtures (Wood's metal) with low melting temperature metal while maintaining the callus areas moist. The fracture was tested on an axial-torsion materials testing machine (model AT3045-2800, Bose EnduraTEC Systems Corp., Eden Prairie, MN) with a low-capacity 50 in-lb torque cell (Transducer Techniques, Temecula, CA).The femur was aligned so that the longitudinal axis of the mid-femur was approximately colinear with the longitudinal axis of the proximal and distal blocks of potting material and torsional actuator of the materials test machine. The fractures were loaded to failure in torsion at a rate of 0.5 degree/s. Thereafter, the femurs were stored in 70% ethanol at 10°C until further examination.

Torques was measured to within ±0.002 Nm in the 1.14 Nm range, and angles were measured to within 0.25% (0.15°) of the full scale (60°). Torque and rotation were continuously recorded over three consecutive cycles with a personal computer and WinTest software (Bose EnduraTEC Systems Corp.) at a rate of 20 Hz. The data were translated into a load–deformation curve where ultimate torque (maximum load) and ultimate stiffness (equal to the maximum slope of the load–deformation curve) were calculated. The energy absorptive capacity was measured as the area under the load–deformation curve until ultimate torque. The yield point was defined as the point at which a 20% reduction in maximum slope occurred.

Dual-Energy X-ray Absorptiometry (DEXA)

For all fractures, only one fracture line and no loose fragments were observed. The two ends of bones tested were realigned at their original position. The femurs were placed in 70% ethanol with the anteromedial surface downward and scanned by DEXA using the regional high resolution analysis program for small animals (Discovery; Hologic, Inc., Waltham, MA). Bone mineral content (BMC) around the fracture line was measured in an 8.0-mm high diaphysial segment (4 mm proximal and 4 mm distal to the fracture line).

Statistical Analysis

The data were tested for normality and homogeneity of variance and when these conditions were fulfilled, parametric analyses were applied. Otherwise, nonparametric analyses were used.

The effects of the different exposures were evaluated using the following groups: nicotine, tobacco extract, tobacco extract plus nicotine, and vehicle (saline). Differences between these groups were tested by one-way analysis of variance (ANOVA) or a Kruskal-Wallis test. In cases in which differences occurred, all pair wise multiple comparisons procedures were applied (unpaired *t*-test or Mann-Whitney Rank Sum Test). Changes in weight in the individual groups during the experiment were analyzed by using unpaired *t*-test as normality and homogeneity of variance were fulfilled.

The value of $p < 0.05$ (two tailed) was considered statistically significant.

RESULTS

Out of 104 animals operated on, 89 were included in the subsequent experiment. Seven animals died as a result of anesthesia: six as a consequence of incomplete fracture, and two because of pump malfunction. The number of animals in each group was: nicotine ($n = 22$), tobacco extract ($n = 23$), nicotine plus tobacco extract ($n = 22$), and vehicle (saline) ($n = 22$).

The results of the mechanical testing of the fractured femurs are given in Figure 1. After 21 days of healing, the ultimate torque of the fractures was decreased only in the group given tobacco extract alone, both in comparison with the nicotine group and the vehicle (saline) group (20%, $p = 0.023$ and 21%, $p = 0.010$, respectively). No difference in ultimate torque was found between the tobacco extract group and the tobacco extract plus nicotine group (10%, $p = 0.239$). Ultimate stiffness and energy absorption capacity revealed no differences between any of the groups. Compared with the nicotine group and vehicle (saline) group, the torque at yield point in the tobacco extract group was decreased by 26% ($p = 0.004$) and 23% ($p = 0.056$), respectively. An 18% ($p = 0.013$) reduction in torque at yield point was observed in the tobacco extract plus nicotine group compared with the nicotine group, as was a 14% reduction when compared with the vehicle (saline) group ($p = 0.167$). Again, no difference was found between the tobacco extract group and tobacco extract plus nicotine group (10%, $p = 0.286$).

Serum nicotine levels at fracture, 2 weeks of healing, and at killing are given in Table 1. The mean serum concentrations of nicotine in the nicotine group and the tobacco extract plus nicotine group were in the range of 39–49 ng/mL during the observation period, and those of the nicotine metabolite cotinine were 255–320 ng/mL. Nicotine was not detected in the vehicle (saline) or tobacco extract groups. The anatabine concentration in the tobacco extract prior to its 1:30 dilution was 1284 ng/mL.

BMC is given in Figure 2. No significant differences in callus DEXA-BMC were observed between the four groups at killing.

The mean body weights in each group at the beginning and the end of the study are given in

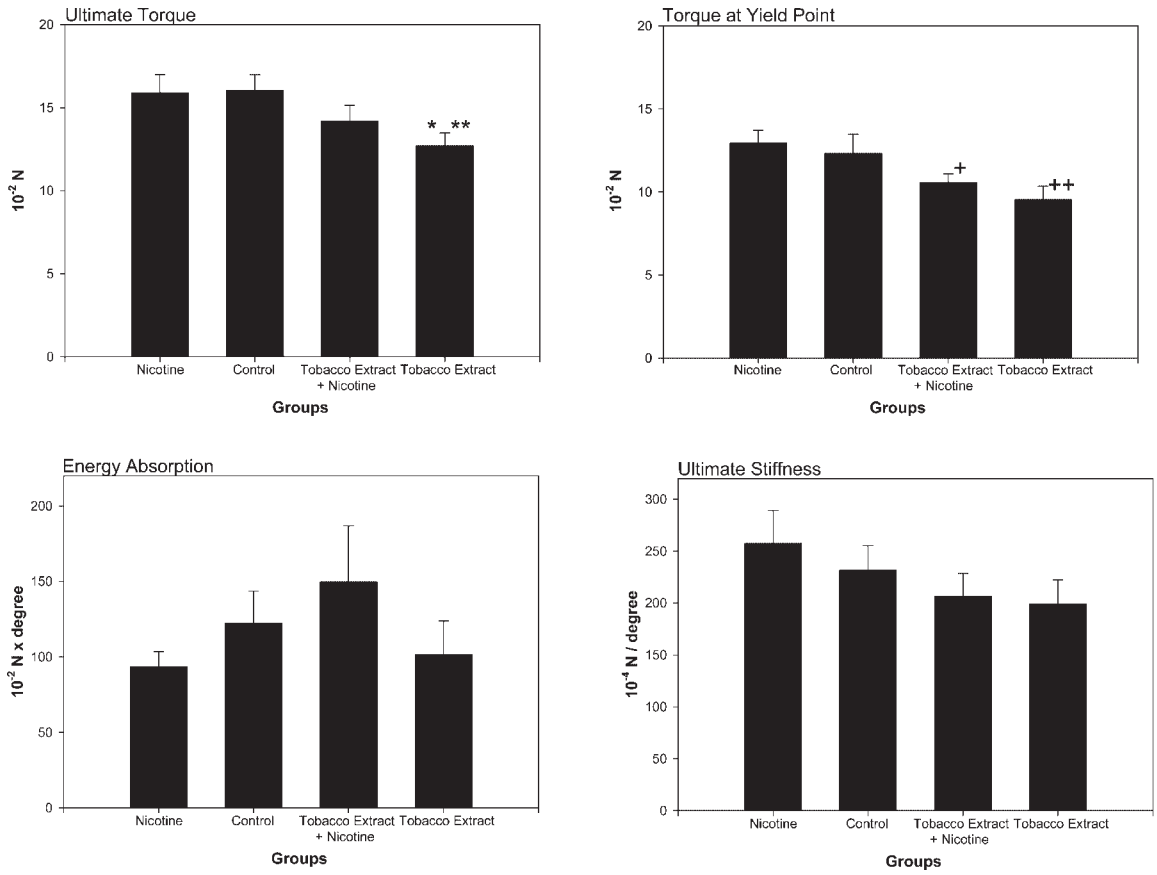

Figure 1. Mechanical properties of the fractured femur after 3 weeks of healing. Graphs showing ultimate torque, torque at yield point, energy absorbed and ultimate stiffness. Ultimate torque was significantly lower in the tobacco extract group compared with the nicotine group and the control group ($^{*}p = 0.023$ and $^{**}p = 0.010$). Torque at yield point was significantly decreased in the tobacco extract plus nicotine group compared with the nicotine group ($^{+}p = 0.013$) and the tobacco extract group compared with the nicotine group ($^{++}p = 0.004$) and. Data are expressed as mean ± SEM.

Table 2. No differences in body weight were found between the groups at the day of pump implantation. All animals gained weight during the study. At fracture in the nicotine group, the body weight was 4% lower compared with the vehicle (saline) group ($p = 0.03$). However, (at killing) no difference in body weight was found between the nicotine group and the vehicle (saline) group. At fracture,

Table 1. Serum Concentrations of Nicotine and Cotinine during the Experiment

	Vehicle	Nicotine	Tobacco Extract	Tobacco Extract plus Nicotine	Between Nicotine Groups
Nicotine (ng/mL)					
Fracture	0 ± 0	43.36 ± 1.60	0 ± 0	41.95 ± 1.87	0.570
2 weeks of healing	0 ± 0	46.18 ± 1.31	0 ± 0	38.73[a] ± 1.95	0.003
3 weeks of healing	0 ± 0	49.14 ± 2.48	0 ± 0	47.64 ± 3.01	0.702
Cotinine (ng/mL)					
Fracture	0 ± 0	320.82 ± 10.51	0 ± 0	310.55 ± 8.74	0.456
2 weeks of healing	0 ± 0	264.50 ± 4.67	0 ± 0	255.32 ± 9.91	0.407
3 weeks of healing	0 ± 0	274.14 ± 8.25	0 ± 0	285.55 ± 12.18	0.442

Mean values ± SEM.
[a]Significantly different from nicotine, $p < 0.05$.

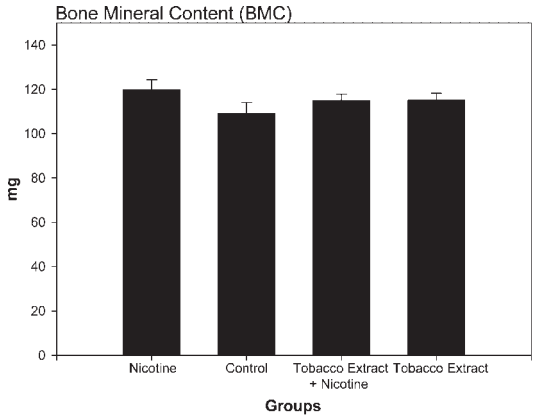

Figure 2. Bone mineral content of the fractured femur after 3 weeks of healing. BMC are measured in an 8-mm high segment located around the fracture line. No significant differences among the groups were observed. Data are expressed as mean ± SEM.

the tobacco extract group and the tobacco extract plus nicotine group had an increase in body weight compared with the vehicle (saline) group (7%, $p < 0.001$ and 5%, $p < 0.001$, respectively). At killing, no differences in body weight were seen between the tobacco extract plus nicotine group and vehicle (saline) group, whereas the body weight of the tobacco extract group was increased by 5% compared with the vehicle (saline) group ($p = 0.01$). No differences in consumption of tobacco extract were seen between the tobacco extract group and the tobacco extract plus nicotine group (28.3 ± 1.2 mL and 25.3 ± 2.4 mL, respectively). Mean of total volume of fluid consumed per 24 h for all treatment groups ranged from 56.6 to 61.7 mL.

DISCUSSION

The present study shows that tobacco extract impairs the mechanical strength of the healing fractures in this model, whereas administration of nicotine does not seem to influence the mechanical strength, when administered in a dose inducing serum nicotine levels similar to those observed in daily smokers. Our nicotine data correspond with Abulencia et al.,[6] who showed that rats treated with the same amount of nicotine as used in our experiment (3 mg/kg/day) had normal mechanical strength of healing fractures after 3 and 6 weeks. Abulencia et al. also used a threefold higher dose of nicotine and this treatment induced a decrease in fracture strength after 3 weeks of healing. However, this higher dose of nicotine did not influence mechanical strength after 6 weeks of healing.

Raikin et al.[7] treated rabbits with nicotine in a dose of 9 mg/kg/day and found that the mechanical strength of a mid-shaft tibial osteotomy was decreased by 34% after 8 weeks of healing. Spine fusion in rabbits treated with nicotine (6 mg/kg/day) was investigated after 5 weeks of healing.[8] Nicotine did not influence the mechanical strength of the fused spine. In both studies, the administration of nicotine began at the initial surgery and the used dose of nicotine was two to three folds higher than the dose used in our study.

Daftari et al.[14] investigated the effect of nicotine (9 mg/kg/day) on revascularization of rabbit autologous cancellous bone implanted in the anterior eye chamber for 2 and 4 weeks. Nicotine treatment delayed the revascularization and augmented necrosis within the graft.

In addition, nicotine in a dose of 3 mg/kg/day has been shown to decrease the expression of a wide range of cytokine genes associated with neovascularization and osteoblast differentiation (VEGF, BMP-2, BMP-4, BMP-6, bFGF, and collagen I).[15] Thus, varying results have been reported on the effects of nicotine on bone fracture healing, some of which could be ascribed to the difference in nicotine doses or the difference in the experimental animal species studied.

The effects of tobacco extract on bone formation have also been investigated in vitro. Galvin et al.[23]

Table 2. Body Weight during the Experiment

	Vehicle	Nicotine	Tobacco Extract	Tobacco Extract plus Nicotine	*p* Value
Body weight (g)					
Pump implantation	253.0 ± 1.3	252.5 ± 1.7	252.8 ± 1.3	254.7 ± 1.6	0.698
Fracture surgery	307.0 ± 2.7	296.2 ± 2.1	328.2 ± 2.5	321.7 ± 2.4	<0.001
3 weeks of healing	362.1 ± 4.7	365.0[a] ± 5.2	379.0 ± 3.8	370.9 ± 3.7	0.038

Mean values ± SEM.
[a]Significantly different from tobacco extract, $p < 0.05$.

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cultured chick embryonic tibiae in tobacco extract (with nicotine) and nicotine alone. They found that tobacco extract markedly depressed bone collagen synthesis. Nicotine alone also hampered collagen synthesis. However, the nicotine had to be present in a 12-fold higher concentration to induce the same depression in collagen synthesis as the one observed when using tobacco extract. Lenz et al.[24] cultured osteoblast-like cells obtained from chick embryo calvarias in a medium containing increasing concentrations of tobacco extracts (with nicotine). They found that collagen synthesis decreased with increasing concentrations of tobacco extract. They compared the decrease with tobacco extract to their previously published data on nicotine alone,[25] and concluded that nicotine had to be present in at least a threefold higher concentration to induce the same depression in collagen synthesis. These in vitro studies indicate that nonnicotine components of the tobacco may impair fracture healing.

Our in vivo study is to be interpreted within the confines of the experimental model, and the nicotine and tobacco extract administration schedule and route. The closed fracture in the rat, as described by Bonnarens and Einhorn,[21] is often used, as it produces a uniform cross-section fracture, which allows comparison of the fracture to other studies. However, the technique does not completely mimic a normal fracture scenario, due to the prefracture stabilization with an intramedullary K-wire. It also bears emphasizing that bone healing in the rat is different from bone healing in humans, as the diaphyseal cortical rat bone does not have Haversian osteons.

Axial torsion tests were performed to determine mechanical strength, stiffness, and energy to failure. Mechanical strength was reduced to a greater degree in the tobacco extract group compared with the nicotine group and vehicle group. Ultimate stiffness and energy absorption to failure were also reduced, but not significantly. The lack of significance for stiffness and energy absorption may be due to their larger variances compared to the variances of ultimate torque and torque at yield point.

Our primary outcome measures for identifying relative effects of nicotine and tobacco extract on fracture healing were the mechanical tests. Whereas histological and histomorphological analyses of the healing fractures would provide additional information on which to judge the effects of nicotine and tobacco extract, we chose to concentrate on mechanical performance as the measure most closely related to clinical function. Because mechanical failure testing is destructive, either an additional number of animals for histologic material would have been required, or we would have needed to perform histologic analysis on bone and callous tissue that had already undergone failure testing.

In regard to the biology of fracture healing,[26] Lenz et al.[24] and Galvin et al.,[23] as described, reported that tobacco extract without nicotine hampers collagen synthesis in vitro to a greater extent than does nicotine alone. This corresponds with the biomechanical findings in the present study.

The effects of prefracture exposure time and postfracture healing time were not evaluated in this study. We exposed the rats to the nicotine and/or tobacco extract for 1 week prior to fracture, and during the following 3-week healing period. The 1-week prefracture exposure was chosen to equilibrate the concentrations of nicotine and tobacco extract in the plasma and body tissue. The 3-week postfracture period allowed us to avoid full consolidation of the healing fracture in the rat.

An additional variable factor of unknown effect is the route of administration. In this study, the nicotine was administered by an osmotic pump.[27] The serum nicotine levels achieved with this route of administration were similar to those seen in patients who smoke one to two packs of cigarettes a day. The tobacco extract in this study was delivered as an aqueous extract of noncombusted tobacco, and was administered orally. The aqueous tobacco extract was a reasonable first choice. Because the pH in the mouth is fairly neutral, an aqueous solution is what the oral cavity would must likely absorb during smoking (other than pyrolosis products from burned tobacco). It is recognized that this exposure differs from that of cigarette smokers who are exposed to the tar fraction of cigarette smoke. However, exposure to orally ingested tobacco extract is probably similar to exposure experienced with smokeless tobacco products such as chewing tobacco or snuff.

As mentioned, there are about 3500 compounds in the particulate phase of cigarette smoking, and one cannot expect that the aqueous tobacco extract will contain all components potentially extracted from cigarette tobacco. As a surrogate for the measure of all compounds in the tobacco, we measured anatabine a minor alkaloid in the tobacco extract in a pilot study performed separately but with a setup similar to that of the present study. This marker was measured in the aqueous tobacco extract and in 24-h urine samples, from rats in metabolic cages. The urine anatabine data showed (data not shown) that exposure were continuous over the 4 weeks of exposure. An overall mean of 15 ng/mL

was measured in this small sample, corresponding to the anatabine levels measured in urine in Caucasians who smoke 20 cigarettes per day.[20,28]

The tobacco extract potency may have changed over time as the decay of the different compounds in the aqueous tobacco extract is dissimilar. Again, measuring serum levels of different tobacco extract markers would still only be a surrogate for the 3500 compounds in tobacco. Furthermore, it is unknown whether the rate of decay in rats is different from that of humans. Precisely which compounds in the tobacco extract are potentially inhibitory, leading to impaired strength of the healing fracture, is, to our knowledge, unknown and beyond the scope of this study.

Although these data require confirmation with careful manipulation of nicotine and tobacco extract doses and exposure periods, the results of such studies may prove important in determining the relative safety of nicotine replacement therapy compared to smoking, or of various types of tobacco products, in patients with fractures.

In conclusion, our study shows that tobacco extract, but not nicotine, at clinically relevant doses, decreases mechanical strength of healing rat femoral fractures, when administered for 1 week prior to and 3 weeks after the fracture. Further studies defining the tobacco extract component(s) responsible, and the influence of exposure period and dose, and evaluated by supportive histological and histomorphological analyses, may be helpful in assessing the risks of various tobacco products with regard to fracture healing.

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