Influence of Maternal Nicotine Exposure on Neonatal Rat Bone: Protective Effect of Pentoxifylline

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Limited research in young adults and immature animals suggests a detrimental effect of tobacco on bone during growth. The aim of this study was to determine the adverse effects of maternal nicotine exposure during pregnancy and lactation on neonatal rat bone development, and to determine a protective effect of pentoxifylline (PTX). Gravid rats were assigned into four groups, one control (group I) and three experimental (groups II, III, and IV). In group II, pregnant rats received 3 mg/kg/day nicotine alone, subcutaneously, until 21 days postnatal. In group III, pregnant rats received nicotine (3 mg/kg/day) and PTX (60 mg/kg/day). In group IV, pregnant rats received PTX alone (60 mg/kg/day). Whole body mineral density (BMD), content (BMC), area (BA), and histopathologic and morphologic findings of the femur were determined at 21 days of age. The study revealed that nicotine exposure (group II) decreased birth weight, pregnancy weight gain, and length of femur compared with other groups ($P < 0.01$). Birth weight was higher in groups III (PTX + nicotine) and IV (PTX) than in group II (nicotine). Body weight at 21 days of age was higher ($P = 0.009$) in the PTX alone group (group IV) compared with the other groups. BMD was higher ($P < 0.001$) in the PTX-treated groups (group III and IV) compared with other groups. In addition, there were more apoptotic chondrocytes in the hypertrophic zone of rats exposed to nicotine alone (group II) compared with the other groups ($P < 0.001$). In conclusion, maternal nicotine exposure resulted in decreased birth weight, pregnancy weight gain, and bone lengthening, and increased apoptosis. Pentoxifylline supplementation was found to prevent the adverse effects of maternal nicotine exposure on BMD and birth weight. Exp Biol Med 232:398–405, 2007

Key words: maternal; nicotine exposure; rat bone; pentoxifylline

Introduction

The growth plate is the final target organ for longitudinal growth, which results from chondrocyte proliferation and differentiation. During the first year of life, longitudinal growth rates are high, but this is followed by a decade of modest longitudinal growth (1). Prenatal exposure of human fetuses to tobacco smoke through maternal passive smoking has been epidemiologically linked to reduced birth weight, enhanced susceptibility to respiratory diseases, and changes in the immune system (2). Limited research in young adults and immature animals suggests a detrimental effect of tobacco on bone during growth (3). Epidemiological studies have focused on the deleterious effects of smoking on human health. It has been well documented that cigarette smoke decreases bone mineral density (BMD) (4, 5) and increases the risk of bone fracture (6). Despite the knowledge that cigarette smoking is a well-established risk factor, the mechanism responsible for the adverse effects of cigarette smoke on bone has not been determined exactly. Collectively, the literature suggests that the toxic effect of cigarette smoke may be mediated through a deficiency in estrogen levels.

A number of in vitro studies have indicated that in osteoblasts, elevated levels of intracellular cyclic AMP (c-AMP) enhance their bone-forming activity (7). Kimmel *et al.* (8) and Shen *et al.* (9) in 1993 observed that c-AMP and c-AMP–dependent protein kinase may be the primary initiators of the growth response of rat bone to intermittent pulse of parathyroid hormone (PTH). In addition to the rate of synthesis of c-AMP by adenylate cyclase, c-AMP levels can be regulated by the rate of hydrolysis to the inactive form of 5′ AMP by cyclic nucleotide phosphodiesterases

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(PDEs). The PDEs are a large group of enzymes consisting of at least nine groups of isoenzymes encoded by distinctive genes and expressed in various tissues in a tissue-specific manner (10, 11). Phosphodiesterase inhibitors are effective in elevating intracellular c-AMP levels by inhibiting the breakdown of c-AMP by PDEs. Therefore, administration of PDE-inhibiting compounds such as pentoxifylline (PTX) might have the potential to increase bone mass by elevating intracellular c-AMP levels.

The aims of this study were to determine and quantify the adverse effects of maternal nicotine exposure during pregnancy and lactation on neonatal rat bone development, and to establish whether PTX, a methylxanthine derivative and an inhibitor of c-AMP PDEs, protects the neonatal rat bone against the adverse effects of maternal nicotine exposure.

Materials and Methods

Appropriate permits for the study were obtained and the experiments conformed to the Council Directive of the European Communities.

Male and female adult white Sprague-Dawley virgin rats weighing 170–200 g were purchased from the Medical Science Research Center of Erciyes University. The study was performed in September 2005. The rats were acclimatized to caged laboratory conditions and were allowed to feed with standard pellets during the study. Rats were housed in stainless steel cages at room temperature in a humidity-controlled room with a 12-hr light/dark reversed schedule (lights on between 0700 and 1900 hrs). The animals were fed with a stock diet (rat food; Aytekinler, Ankara, Turkey; protein 24%, cellulose 7%, ash 8%, metabolic energy 2.65 kcal/g, calcium 1%, and phosphorus 0.9%). Although the food intake of the animals in the various groups was not measured, there was no clear evidence that it varied between groups. Pregnant rats were obtained by mating virgin females overnight with a sexually experienced male. We checked the plug sign every day, and pregnancy was confirmed by the presence of a vaginal plug of semen in the breeding cage the following morning. After confirming pregnancy with vaginal smear method (12), gravid rats (dams) were then randomly assigned into four equal groups, one control ($n = 10$, group I) and three experimental groups (group II, III, and IV). In group II ($n = 10$), pregnant rats received 3 mg/kg/day nicotine [(–)-nicotine tartarate, Sigma Chemical Co., St. Louis, MO] subcutaneously (sc) until 21 days postnatal. In group III ($n = 10$), pregnant rats received nicotine (3 mg/kg/day) and a simultaneous and separate injection of 60 mg/kg/day pentoxifylline (Trental 100 mg/5 ml ampule, Hoechst Marion Roussel, Inc., Frankfurt, Germany). In group IV ($n = 10$), pregnant rats received 60 mg/kg/day pentoxifylline alone. The dose of PTX used in the study is closer to the dose of PTX commonly used in humans (10–20 mg/kg/day) and was shown to decrease the side effects such as trembling and prostration in the animals (13). Control dams were injected saline solution sc daily during pregnancy and lactation for the same period. Groups I, II, and IV also received an injection with placebo. Nicotine was administered sc with 1-ml tuberculin syringes, to the mother only, which implies that it reached the fetuses and the neonates only via the placenta and mother's milk. Clinical status of the dams was monitored daily during nicotine treatment. All dams were allowed normal delivery during the 20th or 21st day of gestation, and maternal and offspring body weights were all recorded in each group at birth and 21 days of age.

All pup rats underwent dual energy x-ray absorptiometry (DEXA) using a Hologic QDR 4500 Elite apparatus (Hologic, Inc., Waltham, MA) at 21 days of age. During the study, the animals were anesthetized with intraperitoneal ketamine (50 mg/kg) to keep them immobilized. Animals were placed lying ventrally, and then a whole body scan was carried out. The images obtained were analyzed with the software package Rat Whole Body Version 8.26a:3 (Hologic). After the DEXA study, the animals were sacrificed by exsanguinations through an abdominal aortic puncture. The blood sample was kept for laboratory determinations. Carcasses were dissected to obtain the femurs. Bone tissue was fixed in 10% formaldehyde, decalcified, embedded in paraffin, and sectioned at a thickness of 6 μm. The mounted sections were stained with hematoxylin and eosin. The sections were photographed with an Olympus BX50F-3 microscope (Olympus Optical Co., Tokyo, Japan). Each sample was measured five times, blindly. The concentrations of serum calcium, phosphorus, and alkaline phosphatase of the offspring were determined. Bone variables were evaluated including whole bone mineral content (BMC; g), BMD (g/cm^2), and area (BA; cm^2). Femur length, width of the epiphyseal plate (μm), width of the hypertrophic zone (μm), and number of total apoptotic chondrocytes in the hypertrophic zone were determined in each group. The widths of the epiphyseal plate and hypertrophic zone were determined, as described in a previous study (14). We used three slides per animal. The means of hypertrophic zone and width of the epiphyseal plate of five different areas were measured using the oculometric analysis method (Fig. 1). Overall growth plate length was measured by region centered on the long axis of the femur. Horizontal lines were drawn along the contours of both the epiphysis on the proximal side of the growth plate and the chondro-osseous junction on the distal side of the growth plate. Individual zonal length was measured for the hypertrophic zone. Hypertrophic zonal mean lengths and width of the epiphyseal plate of five different areas were measured by using the oculometric analysis method. The measurements of each area were first averaged for each and then the average of all the slides was used to calculate true means hypertrophic zonal mean length and width of the epiphyseal plate for each animal.

Analyzed parameters were interpreted as mean ± standard deviation (SD). The difference between the groups

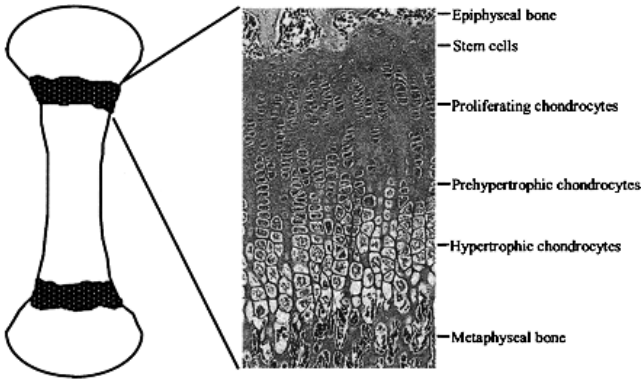

Figure 1. Functional organization of the growth plate (1). Data from van der Eerden *et al.* (1).

was evaluated by one-way ANOVA test. *Post hoc* evaluation of the statistically significant parameters was done by Scheffe test. In all tests the P value <0.05 was accepted as significant. All statistical analyses were done by using SPSS 10.0 software for Windows (SPSS Inc., Chicago, IL).

Results

Clinical Results. Some signs of distress and irritability such as hyperactivity and anger were observed immediately after nicotine administration in nicotine-exposed experimental dams. Ten dams per group were treated. However 2, 2, and 1 dams with their pups died within the 8 days after confirming the pregnancy in groups II, III, and IV, respectively. For this reason, they were excluded from the study, and 10, 8, 8, and 9 dams with their litters were included in the groups I, II, III, and IV, respectively. The numbers of offspring rats per animal were 7.9 ± 3.6, 8.6 ± 2.7, 8.6 ± 2.9, and 10.2 ± 1.8 in the groups I, II, III, and IV, respectively ($F = 0.863$, $P = 0.469$). There was no death within the postnatal 21 days. The birth weight and body weights of the rat pups on postnatal day 21 are shown in Table 1. The mean birth weight of the rat pups exposed to nicotine alone (group II; 5.47 ± 0.39 g) was lower than those of group III (nicotine + PTX; 6.22 ± 0.25 g, $P = 0.003$), group IV (PTX; 6.74 ± 0.31 g, $P = 0.001$), and group I (control; 6.38 ± 0.63 g, $P = 0.002$). The mean body weight of the rat pups treated with PTX alone (group IV; 57.40 ± 6.64 g) was higher than that of groups I, II, and III (49.77 ± 4.34 g, 47.94 ± 4.96 g, and 49.94 ± 7.17 g, respectively) on postnatal day 21 ($F = 6.785$, $P = 0.009$). However, the rate of body weight gain during lactation was not different between groups I, II, III, and IV (7.9 ± 2.6, 8.5 ± 2.2, 8.1 ± 1.1, and 8.5 ± 1.0 times of birth weights, respectively, $F = 0.260$, $P = 0.854$). Although means of maternal weight before gestation of the groups were similar ($F = 0.248$, $P = 0.895$), mean maternal weight at delivery in control (group I; 275 ± 14 g) was higher than that of the mothers exposed to nicotine alone (group II; 255 ± 25 g, $P = 0.009$, Table 1).

Histopathologic and Morphologic Results. The length of femur increased in control (group I; 21.1 ± 0.3 mm) and in the rat pups treated with PTX alone (group IV, 20.9 ± 1.3 mm) compared with the rats exposed to nicotine (group II; 19.1 ± 1.6 mm; $P < 0.001$) and nicotine + PTX (group III, 19.2 ± 1.2 mm, $P < 0.001$; Table 2). The means width of the epiphyseal plate and hypertrophic zone of the groups were similar to each other (Table 3) . The number of apoptotic chondrocytes in the hypertrophic zone was significantly increased in the rats exposed to nicotine (group II; 7.1 ± 1.6) compared with group III (nicotine + PTX; 3.9 ± 1.1, $P < 0.001$), group IV (PTX; 4.1 ± 1.1, $P < 0.001$), and group I (control; 4.3 ± 1.4, $P < 0.001$, Table 3 and Fig. 2).

The Results of Bone Variables. The values of BMD, BMC, and BA in the groups are shown in Table 2. The mean BMD of rat pups treated with PTX alone (group IV; 0.086 ± 0.005 g/cm^2) and rats treated with nicotine and PTX (group III; 0.086 ± 0.005 g/cm^2) were higher than those of the control (group I; 0.079 ± 0.003 g/cm^2, $P < 0.001$) and the rats treated with nicotine alone (group II; 0.077 ± 0.003 g/cm^2, $P < 0.001$; Fig. 3). The value of BMC and BA of groups I, II, III and IV were similar to each other ($P > 0.05$). The BMD data expressed as BMD/body weight in groups I, II, III, and IV were 1.60 ± 0.12, 1.62 ±

Table 1. Influence of Maternal Exposure to Nicotine, PTX, and Nicotine + PTX During Pregnancy and Lactation on the Body Weight[a]

Characteristics (g, mean ± SD)	Control (group I) $n = 79$ pups, 10 litters	Nicotine (group II) $n = 69$ pups, 8 litters	Nicotine + PTX (group III) $n = 69$ pups, 8 litters	PTX (group IV) $n = 91$ pups, 9 litters
Maternal weight before gestation	183 ± 12	183 ± 22	181 ± 18	183 ± 11
Maternal weight at delivery	275 ± 14[b]	255 ± 25	264 ± 19	265 ± 21
Birth weight of the offspring	6.38 ± 0.63	5.47 ± 0.39[c]	6.22 ± 0.25	6.74 ± 0.31
Body weight of the offspring on day 21	49.77 ± 4.34	47.94 ± 4.96	49.94 ± 7.17	57.40 ± 6.64[d]

[a] PTX, pentoxifylline.
[b] Difference is significant compared with group II.
[c] Difference is significant compared with groups I, III, and IV.
[d] Difference is significant compared with groups I, II, and III.
*$P < 0.05$ is statistically significant.

Table 2. Influence of Maternal Exposure to Nicotine, PTX, or Nicotine + PTX During Pregnancy and Lactation on the Bone Variables

Bone variable (mean ± SD)	Control (group I) $n = 79$ pups, 10 litters	Nicotine (group II) $n = 69$ pups, 8 litter	Nicotine + PTX (group III) $n = 69$ pups, 8 litters	PTX (group IV) $n = 91$ pups, 9 litters
Bone mineral density (BMD) (g/cm^2)	0.079 ± 0.003[a]	0.077 ± 0.003[a]	0.086 ± 0.004	0.086 ± 0.005
Bone mineral content (BMC) (g)	1.313 ± 0.258	1.291 ± 0.219	1.341 ± 0.189	1.320 ± 0.179
Bone area (BA) (cm^2)	16.5 ± 3.1	16.38 ± 2.7	15.7 ± 3.5	15.4 ± 3.9
Femur length (mm)	21.1 ± 0.3[b]	19.1 ± 1.6	19.2 ± 1.2	20.9 ± 1.3[b]

[a] Difference is significant compared with groups III and IV.
[b] Difference is significant compared with groups II and III.
*$P < 0.05$ is statistically significant.

0.21, 1.70 ±0.31, and 1.51 ± 0.26 BMD/kg, respectively ($F = 0.542$, $P = 0.112$). There were no significant differences in the serum levels of calcium, phosphorus, and alkaline phosphatase of the groups (data not shown).

For specific analyses, the groups were subdivided into groups of male and female rats. There were no significant differences between the subgroups for the all parameters included in the study (data not shown).

Discussion

Although BMD values were not different between the rats treated with nicotine alone and control, PTX appears to counteract many effects (BMD, birth weight, and apoptosis of chondrocytes in the hypertrophic zone) of nicotine in the study. Our findings are in contrast to the results of Broulik *et al*. (15) who found a significant reduction of BMD and BMC in the nicotine-treated animals compared with animals without nicotine, but in agreement with Iwaniec *et al*. (3) who found no significant nicotine effects on BMC and BMD compared with control. Our study has a limitation. Recommended calcium intake in the rat is 0.5% calcium; higher calcium intake (1%) might have influenced our results and attenuated differences between groups. This study showed that birth weight of neonatal rats exposed to nicotine *via* placenta was less than that of the control rats. There have been studies published in the past confirming an association between maternal smoking during pregnancy and low birth weight (2, 16). Our findings are in contrast with Sheng *et al*. (17) who found that maternal nicotine exposure did not affect either litter sizes or body weights at birth and at 10 days of age. It could be that the pregnancy weight gain was found to be lower in rats exposed to nicotine compared with control in our study, whereas their rats had similar pregnancy weight gain.

Bone mineral density increased in the rat pups given nicotine + PTX (60 mg/kg/day) as well as in those given PTX alone in our study. Kinoshita *et al*. (18) found that administration of PTX (50, 100, 200, and 300 mg/kg/day for 5 weeks) increased the density of x-ray images of the femurs and spinal bones in a dose-dependent manner without changing body weight on densitometric analysis, and the differences were found to be significant compared with a control for the doses of 100, 200, and 300 mg/kg/day. Horiuchi *et al*. (19) injected PTX (5, 25, 50, 100, 200, and 300 mg/kg body weight/day) into the mice sc once a day for 3 weeks from the day of implantation of the bone morphogenetic protein (BMP)-laden disks and found that ossicles from mice treated with >50 mg/kg/day of PTX were significantly larger in size and had a greater calcium content; however, no differences were noted in mice treated with lower doses (5 and 25 mg/kg/day) of PTX. In contrast with our findings, they did not find significant differences in BMD between ossicles in the control and PTX treatment groups. The data from the study of Horiuchi *et al*. (19) indicate that PTX has an anabolic effect on BMP-induced ectopic new bone formation. However, the exact mechanism by which PTX modulates bone formation remains unknown; possible explanations can be deduced from the general pharmacologic actions of this agent. Cells of the osteoblast lineage are central in the process of bone development,

Table 3. Histopathologic Results of Proximal Femur

	Control (group I) $n = 79$ pups, 10 litters	Nicotine (group II) $n = 69$ pups, 8 litters	Nicotine + PTX (group III) $n = 69$ pups, 8 litters	PTX (group IV) $n = 91$ pups, 9 litters
Width of the epiphyseal plate (μm)	716 ± 100	686 ± 116	696 ± 87	721 ± 99
Width of the hypertrophic zone (μm)	419 ± 39	391 ± 33	402 ± 34	411 ± 42
Number of apoptotic chondrocytes	4.3 ± 1.4	7.1 ± 1.6[a]	3.9 ± 1.1	4.1 ± 1.1

[a] Difference is significant compared with groups I, III, and IV.
* $P < 0.05$ is statistically significant.

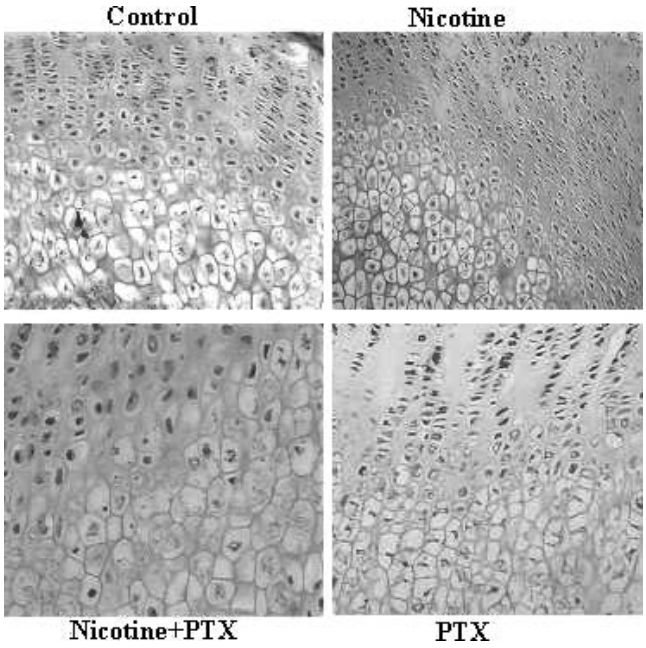

Figure 2. Increased number of apoptotic chondrocytes in nicotine group.

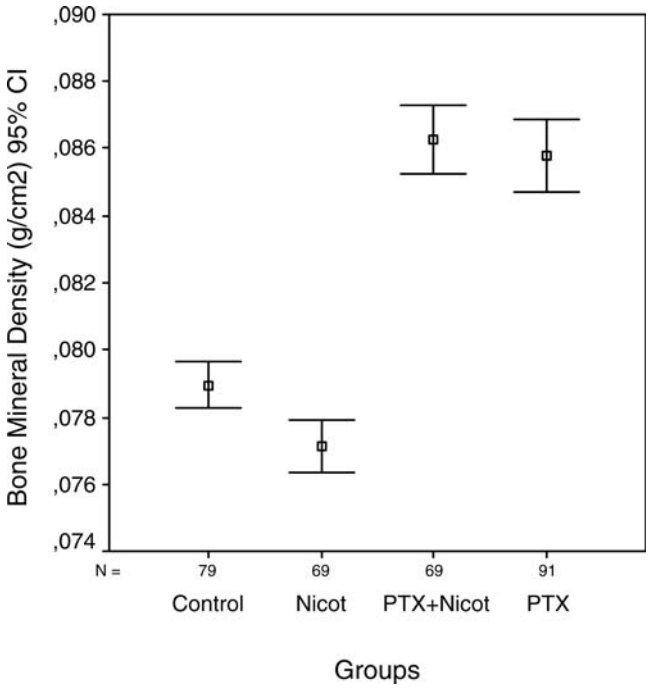

Figure 3. Mean bone mineral density of the groups. Nicot, nicotine; CI, confidence interval.

growth, and remodeling. The development of rapidly dividing osteoprogenitor cells into highly differentiated bone-forming osteoblasts is regulated by a variety of hormones and growth factors. PTH has a well-known physiologic role in bone metabolism and modulates the expression of several genes characteristic of the osteoblast phenotype (20). PTH was regarded as a catabolic hormone that stimulates osteoclastic resorption of bone. However, it is known that intermittent pulses of PTH stimulate bone formation (21). Recently, several reports have shown that an increase in the intracellular levels of c-AMP by PDE inhibitors causes a reduction in the expression of tumor necrosis factor-α (TNF-α), by monocyte-macrophage populations or endothelial cells at a transcriptional level (22). As TNF-α is a cytokine whose biologic actions promote bone resorption and suppress bone formation, it seems plausible that reduced production of TNF-α in marrow as a result of PTX could be a mechanism by which these agents could produce an increase in BMD. One other possible mechanism by which PDE inhibitors might modify bone formation is through the involvement of the signaling pathway for BMP. These proteins have the capacity to induce mesenchymal cells to differentiate into chondrocytic or osteoblastic lineage (23), because high intracellular c-AMP levels in BMP-responding cells amplify BMP effects on the cells and stimulate their differentiation (24), thus promoting bone formation.

The PTX treatment counteracted many effects (BMD, birth weight, and apoptosis of chondrocytes in the hypertrophic zone) of nicotine in the present study. Cigarette use has been identified as a risk factor for low BMD and osteoporotic fracture (4–6). However, the biologic mechanisms by which smoking or components of cigarette smoke influence BMD and bone loss are not well understood. These mechanisms may include local and systemic toxic effects on bone collagen synthesis and alterations in metabolism of adrenal cortical and gonadal hormones (25, 26). Direct toxic effect of tobacco on bone cells (27), decrease of body storage of vitamin D (28), and cellular resistance to calcitonin (29) have also been proposed to explain the effect of smoking. In addition, smoking is associated with an increased concentration of reactive oxygen species and reduced levels of vitamins (30). It has been suggested that reactive oxygen species may be increased in the bone resorption process (31). Rapuri *et al.* (32) have provided some evidences for decreased calcium absorption associated with elevated PTH levels and increased bone resorption in smokers (clearly evident by the increase in bone markers). There is evidence from animal studies to suggest that some component of cigarette smoke damages intestinal villi (33). Nicotine is known to have vasoconstrictive action. However, the subjects were not exposed to cigarette smoke during the study, and thus little or no nicotine effect on the intestinal blood supply would be expected. In human osteoblasts and pulmonary cells of rat, nicotine induced an increase in TNF-α secretion (34, 35). TNF-α can induce apoptosis by mechanisms that involve oxidative and/or genetoxic stress (36). We speculated that the nicotine treatment might increase the TNF-α secretion, and PTX treatment might reverse the effects attributed to nicotine by reducing production of TNF-α. In addition, the rats exposed to nicotine *in utero* might respond to stress by increasing endogen glucocorticoid production,

and this effect might increase the number of apoptotic chondrocytes in the hypertrophic zone in the rats exposed to nicotine in our study. Mehls *et al*. (37) found that production of local insulin-like growth factor-I (IGF-I) was lowered by the corticosteroid *via* IGF-I transcription inhibition, and the rate of apoptosis was also increased, both in growth plate chondrocytes and osteoblast cell lines in rats. The daily nicotine intake of the human males and females, who smoke tobacco, varies between 10.5 and 78.6 mg (38). Assuming that 90% of the nicotine is absorbed on inhalation (39), the nicotine intake of a 60-kg female will be between 0.16 and 1.18 mg/kg body weight per day. The dose of 3 mg nicotine/kg body weight per day used in this study therefore lies within the range of intake of heavy smokers. Although nicotine is the principal pharmacologically active chemical in tobacco, there are several thousand bioproducts of tobacco including toxic oxidant free radicals, carbon monoxide, and hydrogen cyanide (40). These may be more detrimental to bone than nicotine alone.

In agreement with our findings, Ueng *et al*. (41) showed that intermittent inhalation of cigarette smoke delays the bone healing in tibial lengthening. Riesenfeld *et al*. (42) found that bone length was depressed in rats submitted to daily treatment with nicotine for a period of 6 months in spite of the continuous growth of the rats. However, the PTX treatment did not appear to counteract the femur-length effect of nicotine in our study. There is not enough experience about the effect of PTX on bone lengthening in the literature. It is possible that longer treatment periods with higher doses of PTX may be necessary to demonstrate positive femur length effect in rats.

The pharmacologic effect of the PDE inhibitor might be important when considering treatment for fracture repair or diseases associated with systemic bone loss such as osteoporosis (18). Inhibition of PDEs might ultimately result in intracellular accumulation of the cyclic nucleotides and increased levels of c-AMP or cyclic guanosine monophosphate (GMP). Pentoxifylline is a general inhibitor of enzymes, and it has been used in the medical field for many years to improve brain circulation and, more recently, to ameliorate septic shock by aiming at suppression of associated TNF overproduction (43, 44). However, adverse events such as headache and nausea have been associated with PTX treatment (45). Since c-AMP signaling exerts effects in many cells, use of PTX in the developing organism may not be therapeutically feasible for humans. As the PDEs constitute a family of enzymes (isomers), and each isomer is distributed in a tissue-specific manner, identification of the isomer specific to osteoblasts and a selective PDE inhibitor would be desired in order to enhance bone formation without engendering side effects.

Mills published the first report of an adverse effect of maternal smoking on breast-feeding in 1950 (46). Although many studies have not accounted for the sociodemographic differences between women who are smokers and women who are non-smokers (47), large studies have found an association between maternal smoking and a shorter duration of breast-feeding, which remains after adjusting for confounding factors (48). As early as 1933, it was known that nicotine was present in the breast milk of women who smoked (49). The milk-to-plasma ratio for nicotine is 2.9, indicating that nicotine levels in breast milk are almost three times higher than in the mother's blood (50). Nicotine is well absorbed by inhalation in the lungs or through mucous membranes (51), and thus the nicotine in breast milk is well absorbed by the infant. Nicotine ingested in aqueous solution is largely metabolized in the liver before reaching the systemic circulation (52). In breast milk, nicotine is likely to be contained mainly within fat particles; however, the absorption and metabolism of nicotine in breast milk have not been studied (53). In rat studies, it has been found that animals exposed to nicotine had lower levels of prolactin or blockage of prolactin release and impaired lactation compared with controls. Blake and Sawyer state, "Although the smoker's consumption of nicotine would certainly be less than our rat's intake on a per kilogram basis, species differences can make weight-basis comparisons meaningless" (54). However, there seems to be a wide discrepancy between the rat's two sc injections of 0.65-mg nicotine and an adult woman smoking a cigarette, when each cigarette contains approximately 1 mg nicotine (55). Smoking appears to alter breast milk in other ways, such as taste (56), and alters the composition of milk by increasing pro-oxidant effects (57). Although the rate of gain in body weight during lactation was not different than nicotine alone, the birth weight was increased by PTX supplementation in our study. The PTX enters human breast milk after a single oral dose, with a milk:plasma ratio of 0.87 ± 0.62 at 4 hrs (58). Walton *et al*. (59) found that there was no difference in intrauterine pathologic feature between the placebo and treatment groups (PTX 20 mg/kg/day) in their rat study. The possible long-term effects on the rats in the study and on children of mothers who smoke and the effects of PTX supplementation during lactation should be explored in future research.

In conclusion, maternal nicotine exposure during gestation and lactation resulted in decreases in birth weight, bone lengthening, and pregnancy weight gain. Nicotine increased the number of apoptotic chondrocytes in the hypertrophic zone. It may be possible that apoptosis is one mechanism involved in growth retardation. PTX supplementation during gestation and lactation was found to prevent the adverse effects of maternal nicotine exposure on BMD and birth weight. The results of this study suggest that PTX might be a promising agent for smokers in the prevention or treatment of osteoporosis. However, clinical trials as well as further basic studies will be needed to substantiate the efficacy of PTX for these purposes.

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