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Prenatal nicotine exposure retards osteoclastogenesis and endochondral ossification in fetal long bones in rats

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ABSTRACT

This study investigated the mechanisms underlying the retarded development of long bone in fetus by prenatal nicotine exposure (PNE) which had been demonstrated by our previous work. Nicotine (2.0 mg/kg.d) or saline was injected subcutaneously into pregnant rats every morning from gestational day (GD) 9 to 20. Fetal femurs or tibias were harvested for analysis on GD 20. We found massive accumulation of hypertrophic chondrocytes and a delayed formation of primary ossification center (POC) in the fetal femur or tibia of rat fetus after PNE, which was accompanied by a decreased amount of osteoclasts in the POC and up-regulated expression of osteoprotegerin (OPG) but by no obvious change in the expression of receptor activator of NF-κB ligand (RANKL). In primary osteoblastic cells, both nicotine (0, 162, 1620, 16,200 ng/ml) and corticosterone (0, 50, 250, 1250 nM) promoted the mRNA expression of OPG but concentration-dependently suppressed that of RANKL. Furthermore, blocking α4β2-nicotinic acetylcholine receptor (α4β2-nAChR) or glucocorticoid receptor rescued the above effects of nicotine and corticosterone, respectively. In conclusion, retarded osteoclastogenesis may contribute to delayed endochondral ossification in long bone in fetal rats with PNE. The adverse effects of PNE may be mediated via the direct effect of nicotine and indirect effect of maternal corticosterone on osteoblastic cells.

1. Introduction

Demographic and health survey data show that an estimated 10–20% of women smoke during pregnancy in high-income countries (Miyazaki et al., 2015), while 75% of the pregnant women in low- and middle-income countries are exposed to second-hand smoke (Zhang et al., 2015). However, it has been well-documented that tobacco smoking and second-hand smoke exposure during pregnancy are associated with negative consequences including intrauterine growth restriction (IUGR) and its long-term outcomes (Alberg et al., 2014; Qiu et al., 2014). There is a statistically significant negative correlation between nicotine concentrations in meconium and birth weights (Tsinisizeli et al., 2015), demonstrating that nicotine is one of the major components that perturb fetal development (Slotkin et al., 1986).

Nicotine exerts adverse effects on linear growth and development of fetus. Epidemiological reports have shown that maternal smoking reduces the height of newborns (Karatza et al., 2003; Sexton and Hebel, 1984) and that nicotine content in cigarettes is related to reduced birthweight and birth length of fetus (Olsen, 1992). Our previous study revealed that prenatal nicotine exposure (PNE) induced retardation of

Abbreviations: PNE, prenatal nicotine exposure; GD, gestational day; POC, primary ossification center; OPG, osteoprotegerin; RANKL, receptor activator of NF-κB ligand; IUGR, intrauterine growth restriction; GC, glucocorticoid; TRAP, tartrate-resistant acid phosphatase; BSP, bone sialoprotein; DhβE, dihydro-b-erythroidine; α4β2-nAChR, α4β2-nicotinic acetylcholine receptor

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long bone growth of fetus (Deng et al., 2013). Linear growth of long bone develops through endochondral ossification, during which chondrocytes proliferate, maturate and become hypertrophic. Interference with these processes leads to shortening of bone length (Rossi et al., 2002). It is reported that nicotine can act directly on growth plate chondrocytes to decrease matrix synthesis and suppress hypertrophic differentiation, leading to delayed skeletal growth (Kawakita et al., 2008). In addition, our previous work demonstratesd that PNE induced fetal overexposure to maternal glucocorticoid (GC), which induced IUGR (Tie et al., 2016; Xu et al., 2012; Deng et al., 2013). It is possible that PNE may suppress bone development through a direct effect of nicotine and an indirect effect of maternal GC. For the early development of long bone, the distinct condensation of hind limb bud in rodents appears around gestational day (GD) 9 (Taher et al., 2011). Therefore, we hypothesized that PNE might retard bone development in fetus by restricting the process of endochondral ossification. In the present study, we investigated the effects of PNE on the morphology of fetal femurs during GD9-20 to test the possible developmental toxicity of nicotine in bone and explored evidence for the potential mechanisms of these effects.

2. Material and methods

2.1. Animals and treatments

Two-month-old Wistar rats weighing 180–220 g (female) and 260–300 g (male) were obtained from the Experimental Center at the Hubei Medical Scientific Academy (No. 2006–0005, Hubei, China). The rats were housed in a temperature- and humidity-controlled environment on a 12 h light-dark cycle and fed a standard rodent diet. All experiments were performed according to the protocol approved by the Animal Care and Use Committee of the School of Medicine, Wuhan University.

The rats were allowed to acclimate for at least one week before being subjected to experiments. Two female rats were mated with one male rat overnight. The next morning when the evidence of mating (i.e. presence of a vaginal plug or a vaginal smear with sperm cells) was observed was considered as GD 0. From GD 9 to GD20, nicotine was administered subcutaneously to the pregnant rats (n = 12) at a dose of 2 mg/kg per day while rats in the control group (n = 12) received the same volume of saline. On GD20, after the pregnant rats were euthanized, their fetuses were quickly removed from their uterus. Fetal femurs and tibias were harvested and the femoral length was measured.

2.2. Histological analysis

The fetal bone tissues were fixed in 4% paraformaldehyde solution for 24 h, processed, embedded into paraffin, and cut into 6-μm sections. To determine morphologic changes, 4 to 6 sections from different bone samples were stained with H&E. For von Kossa staining, the sections were deparaffinized and stained with 5%$AgNO_3$. For tartrate-resistant acid phosphatase (TRAP) staining, the deparaffinized sections were stained with leukocyte acid phosphatase kit. TRAP-positive cells at the chondro-osseous junction and primary cancellous bone were counted under microscope at 100× magnification.

For immunohistochemical staining, the sections were deparaffinized and hydrated through a graded series of ethanol. After antigen retrieval, endogenous peroxidases were blocked with 3% H_2O_2 for 15 min at room temperature before washed in PBS. After blocked with normal goat serum for 1 h, the sections were incubated with primary antibody overnight at 4 °C. Primary antibodies against Ki67 (ab15580, Abcam) and P57 (sc-8298, Santa Cruz) were used. The corresponding secondary biotinylated antibodies were subsequently incubated for 1–2 hours at room temperature. Immunoreactive proteins were visualized using the avidin-biotin-peroxidase complex, followed by incubation with DAB substrate kit (Dako, Glostrup, Denmark) to display peroxidase activity and color. Apoptosis detection was performed using the DeadEnd Colorimetric TUNEL System (Cat. G7130, Promega, Madison, USA), according to the manufacturer's instructions. To quantify the changes in immunostaining intensity, the mean optical densities were obtained from 10 fields of epiphyseal cartilage from 5 different samples with 2 fields in each sample section.

2.3. Primary chondrocytes culture

The distal femur and proximal tibia were isolated from 1 to 3 day-old Wistar rats as described previously (Zhang et al., 2011). Briefly, the distal femur and proximal tibia were dissected free of soft tissues and kept in cold-PBS on ice before digested with 2.2 mg/ml type II collagenase solution (Invitrogen) incubation at 37 °C for 30 min. After removal of impurities, cartilage was then further digested with fresh type II collagenase solution for 3 h. Chondrocytes were dissociated from collagenase-digested cartilage by pipetting. Cell suspension was then filtered, centrifuged and collected. The chondrocytes described above were seeded in 6-well plates at a density of 4×10^4 cells/well, and cultured in DMEM/F12 with 5% FBS, 100 U/ml penicillin, 100 g/ml streptomycin, and 50 μg/ml of L-ascorbic acid. To determine the effect of nicotine or corticosterone on chondrocytes, after the primary chondrocytes were treated with nicotine (0, 162, 1620, 16,200 ng/ml) and corticosterone (0, 50, 250, 1250 nM), respectively. The concentrations we used above were based on the findings of our recent work (Tie et al., 2016) in which 3.71 ± 0.15 μM (601 ± 24 ng/ml) nicotine and 603 ± 72 ng/ml (1740 ± 208 nM) corticosterone were detected in fetal serum under 2.0 mg/kg per day for PNE. In addition, the treatment duration we used above was based on the study by Paulo and Gyqi who found nicotine affected diverse signaling pathways and regulated global genes expression after treatment for 24 h (Paulo and Gyqi, 2017).

2.4. Primary osteoblastic cells culture

Rat primary calvarial osteoblastic cells were obtained from neonatal rat calvaria as described previously (Shalhoub et al., 1992). The calvaria harvested from 1 to 3 day-old Wistar rats were cut into pieces, and then digested with 2 mg/ml collagenase A and 2.5 mg/ml trypsin for 40 min. After supernatant discarded, the calvaria were further digested with collagenase/trypsin solution for 90 min. The supernatant was filtered and centrifuged to collect preosteoblasts. The cells were seeded in 6-well plates in growth medium (alpha MEM medium containing 10% FBS) until confluence at day 7. The medium was then switched to differentiation medium (alpha MEM medium containing 10% FBS, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid). For the same reason as mentioned in section 2.3, the primary osteoblastic cells were treated with nicotine (0, 162, 1620, 16,200 ng/ml) and corticosterone (0, 50, 250, 1250 nM), respectively, before the cells were harvested 48 h later for further analysis. To confirm the effect of corticosterone or nicotine on osteoblastic cells, the cells were treated with 250 nM corticosterone in the absence or presence of 10 μM mifepristone (glucocorticoid receptor inhibitor), or treated with 1620 ng/ml nicotine in the absence or presence of 1 μM dihydro-b-erythroidine (DHβE), a selective inhibitor of α4β2-nicotinic acetylcholine receptor (α4β2-nAChR). The cells were collected 48 h later and their total RNA was extracted for further analysis.

2.5. Total RNA extraction and gene expression

Total RNA was prepared using Trizol reagent (obtained from Invitrogen Co., Ltd.; Carlsbad, CA, USA) according to the manufacturer's protocol. The tissue samples isolated from the same littermates were pooled for homogenization. A total of 200 ng of purified total RNA was reversely transcribed into cDNA using a First Strand cDNA Synthesis Kit (TransGen Biotech Co., Ltd.). qRT-PCR was performed with an Applied Biosystems. Relative expression of gene was