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Fetal and Neonatal Exposure to Nicotine Disrupts Ovarian Function and Fertility in Adult Female Rats

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Women born to mothers who smoked during pregnancy have been shown to have impaired fertility, although the mechanisms underlying this association are unknown. Nicotine administration in adult animals has adverse effects on the ovary and uterus; however, the effects of fetal exposure to nicotine on postnatal ovarian function have not been determined. The goal of this study was to assess the effect of fetal and neonatal exposure to nicotine on ovarian function and fertility of the offspring. Nulliparous female Wistar rats were given 1 mg·kg^{-1}·d^{-1} nicotine bitartrate, subcutaneously for 14 d prior to mating, during pregnancy and throughout lactation until weaning. Measures of fertility, breeding success, and serum levels of ovarian steroid hormones in offspring were assessed at 4 and 6 mo of age. Fetal and neonatal exposure to nicotine significantly increased the time to pregnancy as the animals aged. Similarly, evidence of altered ovarian steroidogenesis including increased serum progesterone concentrations and a decreased estrogen:progesterone ratio was observed in 6-mo-old animals. We conclude that fetal and neonatal exposure to nicotine results in delayed ovarian dysfunction in adult female offspring.

Key Words: Fetal exposure; infertility; ovarian steroidogenesis; nicotine; time to pregnancy.

Introduction

There is increased concern globally that human exposure to chemicals in the environment results in adverse health outcomes including reproductive disorders and infertility. These concerns are often focused on the impact of environmental contaminants on fetuses, because chemical insults *in utero* have been shown to cause impaired postnatal fertility *(1–5)*. To date most of the research effort on adverse reproductive effects in the offspring has focused on the effects of fetal and neonatal exposure to environmental toxicants with estrogenic activity, such as cholorinated hydrocarbon pesticides, polychlorinated biphenyls, triazine herbicides, and plasticizers *(1,5,6)*. Recently however, there has been an increased interest in postnatal reproductive health following fetal exposure to chemicals encountered through maternal diet and maternal cigarette smoking *(1,7)*.

It has been well documented that cigarette smoking during pregnancy is associated with a number of adverse obstetrical outcomes including miscarriage, stillbirth, placenta previa, placental abruption, premature rupture of the membranes, preterm birth, and low birthweight *(8–11)*. Although cigarette smoking during pregnancy is associated with adverse fetal, obstetrical, and developmental outcomes, 15–20% of all pregnant women smoke during pregnancy *(8,12)*. Furthermore, in human populations there is evidence that *in utero* exposure to cigarette smoke has long-term consequences on the fertility of the offspring. For example, sons born to mothers who smoked during pregnancy have lower sperm counts, decreased fecundability, and reduced numbers of morphologically normal sperm cells *(13–16)* and daughters of mothers who smoked during pregnancy have an earlier menopause, shorter reproductive lifespan, and reduced fecundability *(14,17)*.

Cigarette smoke is estimated to contain as many as 4000 chemicals *(18)* including the addictive component nicotine. Data from animal studies suggest that nicotine exposure may be a critical component in the development of adverse reproductive effects in women who smoke *(19,20)*. However, although there is a clear link between nicotine exposure and adverse ovarian function in adult women, the effects of fetal nicotine exposure on ovarian function and postnatal fertility have not yet been examined. A better understanding of the effects of nicotine is critical because many women who are unable to quit smoking during pregnancy use nicotine-replacement therapy (NRT) as a pharmacotherapy for smoking cessation. While NRT is considered a beneficial treatment for women because it reduces exposure to the thousands of toxic chemicals in cigarette smoke, the effect of the delivery of nicotine from the prenatal to postnatal period of pregnancy is a concern. Therefore, the goal of this study was to identify whether fetal and neonatal exposure to nicotine could alter postnatal fertility and disrupt ovarian steroidogenesis.

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Table 1
Birth Phenotype and Fertility Measures: 4-mo-old Females

n = 6 dams per group	Saline	Nicotine
Time to pregnancy (d)	2.5 ± 0.50	2.2 ± 0.37
Gestation length (d)	22.8 ± 0.25	23.2 ± 0.37
Mating success (%)	100	100
Fertility index (%)	83.3	87.5
Live birth index (%)	98.8 ± 1.18	97.8 ± 1.30
Litter size (*n*)	14.6 ± 0.87	14.3 ± 1.17
Birth weight (g)	6.3 ± 0.08	6.4 ± 0.54
Total litter weight (g)	92.0 ± 2.23	91.4 ± 6.76
Sex ratio (M/F)	1.46 ± 0.58	1.32 ± 0.26
Survival to weaning (%)	100	97.3
Serum estradiol (pg/mL)	24.5 ± 3.29	26.0 ± 1.59
Serum progesterone (ng/mL)	19.4 ± 5.14	20.2 ± 4.28
E_2/P_4 (×1000)	2.36 ± 0.78	1.92 ± 0.45

Table 2
Birth Phenotype and Fertility Measures: 6-mo-old Females

n = 6 dams per group	Saline	Nicotine
Time to pregnancy (d)	2.6 ± 0.51	9.2 ± 1.78[a]
Gestation length (d)	22.2 ± 0.20	22.2 ± 0.17
Mating success (%)	100	100
Fertility index (%)	100	100
Live birth index (%)	98.8 ± 1.18	93.6 ± 2.81
Litter size (*n*)	14.7 ± 0.62	13.7 ± 0.72
Birth weight (g)	6.3 ± 0.07	6.2 ± 0.08
Total litter weight (g)	92.9 ± 5.60	85.1 ± 3.95
Sex ratio (M/F)	1.42 ± 0.50	1.16 ± 0.25
Survival to weaning (%)	93.8	100
Serum estradiol (pg/mL)	26.1 ± 4.16	22.8 ± 1.87
Serum progesterone (ng/mL)	12.7 ± 2.00	18.3 ± 0.81[a]
E_2/P_4 (×1000)	2.27 ± 0.40	1.25 ± 0.10[a]

[a]Significantly different from the saline controls ($p < 0.05$).

Results

Serum Cotinine Concentrations

In dams treated with nicotine, serum cotinine concentrations were 135.9 ± 7.86 ng/mL, whereas cotinine concentrations in the serum of the saline-treated dams were below the minimum detectable limit of the assay. Serum concentrations of cotinine in nicotine-exposed offspring at birth were 26.2 ± 1.78 ng/mL and undetectable in saline-exposed offspring.

Fertility

At 4 mo of age there was no effect of fetal and neonatal exposure to nicotine on time to pregnancy, gestation length, mating success, the fertility index, the live birth index, litter size, birth weight, total litter weight, sex ratio, or survival to weaning (Table 1). Similarly at 6 mo of age there was no significant effect of treatment on mating success, the fertility index, gestation length, sex ratio, litter size, birth weight, and total litter weight (Table 2). Nicotine-exposed offspring tended ($p = 0.12$) to have a lower live birth index than control offspring at 6 mo of age (Table 1). However, the proportion of nicotine-exposed dams that delivered stillborn pups was higher than that of the saline-exposed dams [saline 16% (1 out of 6) vs nicotine 67% (4 out of 6)]. Fetal and neonatal exposure to nicotine significantly increased the time to pregnancy in the offspring at 6 mo of age (Table 2).

Ovarian Steroid Hormone Concentrations

At 4 mo of age there was no effect of fetal or neonatal exposure to nicotine on serum estradiol or progesterone concentrations or the estradiol:progesterone ratio (Table 1). At 6 mo of age, both groups had similar serum concentrations of estradiol, but nicotine-exposed females had greater ($p < 0.05$) serum progesterone and a significant reduction in the estradiol:progesterone ratio (Table 2).

Discussion

This study is the first to demonstrate in an animal model that fetal and neonatal exposure to nicotine, the major addictive component of cigarette smoke, causes impaired fecundability and altered ovarian steroidogenesis in the female offspring. These results are consistent with human epidemiological studies in which maternal smoking has been associated with impaired fertility in the offspring *(14,17)*.

It has been well documented that there is a significant association between adult female smoking and an increased time to pregnancy *(21–29)*. Infertility among women who smoke may be a result of a combination of impaired oocyte function and viability, decreased fertilization rates, altered ovarian steroidogenesis, depleted ovarian reserves, and increased chromosomal abnormalities in oocytes *(30–32)*. More recently, epidemiological studies have suggested that *in utero* exposure to cigarette smoke can result in altered fertility in the female offspring including an increased time to pregnancy *(14,17)*, but the mechanism(s) underlying this observation have not been well defined.

Data from animal studies suggest that of the estimated 4000 chemicals in cigarette smoke, nicotine alone may be responsible for many of the adverse reproductive effects in women who smoke *(19,20,33)*. In adult humans, cotinine, the metabolite of nicotine, has been detected in the follicular fluid of women who smoke *(34–36)* demonstrating that nicotine has access to the ovary and the developing follicles. In pregnant women who smoke or use NRT, nicotine crosses the placenta, concentrates in fetal blood and amniotic fluid, and is detectable in breast milk during lactation *(37)* resulting in both fetal and neonatal exposure to nicotine. We therefore propose that maternal nicotine exposure has direct effects on the developing fetal ovary that are evident later in the reproductive lifespan.

Results from in vivo and in vitro studies have clearly demonstrated that nicotine alone can have adverse effects on ovarian function. For example, in ovarian cell cultures nicotine has been shown to alter estradiol and progesterone production *(19,38–42)*, and nicotine administration in adult rats results in an increase in the number of atretic follicles in the ovary, irregularities in the estrous cycle, impaired ovulation, and altered ovarian steroid hormone concentrations *(19,20,33)*. Similarly, we have demonstrated that fetal and neonatal exposure to nicotine can result in an increased time to pregnancy, which may be a reflection of the altered steroid hormone levels or may represent an overall lengthening of the estrous cycle. Furthermore, we have shown that nicotine exposure results in a higher proportion of dams delivering stillborn pups, all of which are consistent with reproductive aging in rodents.

Results from this study demonstrate a significant effect of nicotine exposure on ovarian function in offspring between 4 and 6 mo of age. Wistar rats reach sexual maturity by approx 4 mo of age, at which time the ovaries become steroidogenically active *(43)*. If nicotine exposure has a deleterious effect on ovarian function, we could expect to see a reduced production of ovarian steroids as a consequence. The increased progesterone secretion, and associated alteration in the estrogen:progesterone ratio may have been due to impaired ovarian development. Another study using reduced protein intake to alter the hypothalamic–pituitary–adrenal axis noted reduced gonadotropin secretion by 70 d of age and significant inhibition of reproductive performance by 1 yr *(44)*. In this study, the nicotine-treated rats had increased serum progesterone and a reduced serum estrogen:progesterone ratio by 6 mo of age. Increased progesterone secretion (and the resultant alteration in the estrogen: progesterone ratio) is known to inhibit pregnancy by interfering with ovulation and by altering cervical mucus *(45)*. It appears that fetal and neonatal exposure to nicotine may negatively affect hypothalamic–pituitary–ovarian function and results in a reprogramming of the ovary, significantly reducing reproductive performance.

Rodents, like humans, exhibit decreased fertility with aging starting at approx 8 mo of age *(46)*. In our study increased time to pregnancy and a trend toward a decreased live birth index was present in 6-mo-old animals that had been exposed to nicotine. We speculate that these results suggest that fetal and neonatal exposure to nicotine resulted in a reprogramming of ovarian function and accelerated reproductive aging in female offspring. This is similar to the model of germ cell death that underlies premature menopause and reduced fecundity in women who smoke *(47)*. These effects may be subtle enough to cause conception delay without producing complete sterility.

The results from this study show that fetal exposure to nicotine has a significant impact on ovarian function and results in both accelerated reproductive aging and impaired reproductive performance in adulthood. Because many couples are choosing to delay having children until a later age *(48)*, premature reproductive aging as a result of perinatal exposure to cigarette smoke may have significant implications for the fertility of women born to mothers who smoked during pregnancy. This study futhers our understanding about the implications of fetal exposure to nicotine and provides information about the mechanisms by which this exposure may contribute to reduced fertility in adulthood.

Materials and Methods

Maintenance and Treatment of Animals

All animal experiments were approved by the Animal Research Ethics Board at McMaster University, in accordance with the guidelines of the Canadian Council for Animal Care. Nulliparous 200–250 g female Wistar rats (Harlan, Indianapolis, IN) were maintained under controlled lighting (12:12 L:D) and temperature (22°C) with *ad libitum* access to food and water. Two weeks prior to mating the dams were randomly assigned to receive either saline (vehicle) or nicotine ($n = 6$ per group). Dams were injected subcutaneously with 1 $mg \cdot kg^{-1} \cdot d^{-1}$ nicotine bitartrate (Sigma Aldrich, St. Louis, MO) or saline for 14 d prior to mating, during pregnancy and post-partum until weaning. Litter size was culled to eight at birth to assure uniformity of litter size between treated (nicotine) and control (saline) litters. To confirm delivery of nicotine to the treated dams, serum cotinine, the major metabolite of nicotine, was measured by ELISA in the dams at weaning and in the trunk blood of culled pups at postnatal d 1 (Cozart Biosciences, Oxfordshire, UK). Cotinine is regarded as the best biomarker of nicotine exposure because it persists longer in the body (20–24 h) than nicotine (2 h). After weaning at postnatal d 21, female offspring were selected randomly and caged as sibling pairs.

Assessment of Fertility

Fertility in the female offspring of the saline- and nicotine-treated dams was assessed in separate groups of animals at 4 and 6 mo of age. Female offspring (F1; $n = 6$ per group at each age) were housed 1:1 with a proven male and monitored daily for confirmation of breeding (i.e., the presence of sperm in the vaginal swab). The day that a positive sign of copulation was observed was designated gestational d 0 (GD0). For each dam (F1), time to pregnancy (i.e., days until detection of sperm in the vaginal swab), gestation length, litter size, litter weight, birthweight, sex, the number of stillbirths, and survival to weaning was recorded. Mating success ([# of females mated/# of females cohabited] ×100), the fertility index ([# of cohabited females becoming pregnant/# of nonpregnant couples cohabited]×100), the live birth index ([# of live offspring/# of offspring delivered] ×100) and the sex ratio (# of male offspring/# of female offspring) were also calculated for animals at both time points.

Steroid Hormone Analysis

In a subset of animals (n = 6 per group at each age), vaginal swabs were performed daily starting at 4 and 6 mo of age to determine the time of estrous. In the morning of estrus, blood samples were collected, allowed to clot at 4°C, centrifuged, and stored at –80°C until analysis. Serum concentrations of estradiol and progesterone were determined by using commercially available radioimmunoassay kits (estradiol: 3rd Generation Estradiol kit, Diagnostic Systems Laboratory, Webster, TX; progesterone: Diagnostic Products Corp., Los Angeles, CA) that have been validated for use with rat serum.

Statistical Analysis

All statistical analyses were performed by Student's *t* test (SigmaStat, v.2.03, SPSS, Chicago, IL). Data were tested for normality as well as equal variance, and when normality or variance tests failed, data were analyzed using the Mann–Whitney rank sum test. Values are presented as mean ± SEM.

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