Hormonal Changes Accompanying Cigarette Smoke-Induced Preterm Births in a Mouse Model

SHEUNG P. NG, BERNARD G. STEINETZ, SALAMIA G. LASANO, AND JUDITH T. ZELIKOFF[1]
New York University School of Medicine, Nelson Institute of Environmental Medicine, Tuxedo, New York 10987

Epidemiologic evidence indicates that maternal smoking increases the risk of preterm birth. While a number of plausible mechanisms for early delivery have been offered, the role of gestational hormones in this smoke-induced outcome is uncertain. Thus, a toxicologic study was performed to examine the effects and underlying hormonal mechanisms of mainstream cigarette smoke (MCS) exposure on gestational duration. Pregnant B6C3F1 mice were exposed by inhalation to MCS for 5 days/week (4 hrs/day) from Gestational Day (GD) 4 to parturition. Smoke-induced effects on gestational length, interpubic ligament length, maternal hormone secretion patterns (estradiol-17β, progesterone, prolactin, and relaxin), body weight gain, postimplantation loss, litter size, and offspring sex ratio were examined. Dams exposed to MCS at a concentration equivalent to smoking less than one pack of cigarettes/day (carbon monoxide = 25 parts per million, total suspended particulates = 16 mg/m^3) demonstrated a significant ($P < 0.05$) shortening of gestational duration (compared with pregnant, air-exposed mice). In addition, MCS-exposed mice sacrificed on GD 18 had significantly ($P < 0.05$) increased interpubic ligament length, elevated serum estrogen levels, and a reduced progesterone to estradiol-17β ratio (compared with air-exposed controls); levels of progesterone and prolactin were only modestly decreased and increased, respectively, in the MCS-exposed mice. Smoke exposure had no significant effects on maternal relaxin levels, body weight gain, postimplantation loss, litter size, or sex ratio. Results of this study demonstrate that inhalation exposure of pregnant mice to a low dose of MCS shortens gestation and alters hormone secretory patterns, which are important for maintaining pregnancy and inducing parturition. These findings support the view that pregnant women who smoke (even modestly) may be at increased risk for preterm birth, and that early delivery may be related (at least partly) to MCS-induced alterations in gestational hormones. Exp Biol Med 231:1403–1409, 2006

Key words: cigarette smoke; preterm birth; pregnancy hormones

Introduction

Of the nearly 6 million American women who become pregnant each year, approximately 20%–35% of them are cigarette smokers (1). Although the hazards of smoking during pregnancy on the fetus and neonate are widely known, 13%–20% of pregnant women continue to smoke during pregnancy (2, 3). Adverse pregnancy outcomes associated with cigarette smoking include preterm birth, intrauterine growth restriction, and premature rupture of the membranes (PROM) (4, 5). Intact cigarette smoke (CS), as well as many of its individual constituents (including nicotine), is also associated with neonatal and childhood complications such as low birth weight, sudden infant death syndrome, and reduced head circumference (5).

Preterm birth (<37 weeks gestational age), which is usually associated with low birth weight (<2500 g), is the single most important determinant of perinatal mortality in the United States (6). Smoking during pregnancy increases perinatal and neonatal mortality by 33% (7). Maternal smoking accounts for up to 14% of all preterm deliveries and for about 20%–30% of all low-birth-weight babies (The American Lung Association: http://www.lungusa.org/site/pp.asp?c=dvLUK9O0E&b=39853). A number of epidemiologic studies have confirmed this latter relationship. For example, Pollack *et al.* (8) demonstrated that smoking during pregnancy was associated with an increased risk of single birth deliveries before 38 weeks for both heavier (>10 cigarettes per day) and lighter (1–10 cigarettes per day) cigarette smokers. A dose-dependent relationship between maternal smoking and spontaneous preterm birth has also been shown in a study examining more than 300,000 live births in Sweden (9). Moreover, an increased risk for preterm labor, PROM, and various other medical complications were observed in a cohort study even when

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[1] To whom correspondence should be addressed at 57 Old Forge Road, Tuxedo, NY 10987. E-mail: judyz@env.med.nyu.edu

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smoking was confined only to the first trimester of pregnancy (10). Toxicological studies also support the association between maternal smoking and preterm delivery. In one study, pregnant mice given a nicotine alkaloid (equivalent to smoking 1.5 packs of cigarettes/day) in the second and third trimesters of gestation demonstrated a significant shortening (compared with the saline-administered controls) of gestational duration (11). However, while the association between cigarette smoking during pregnancy and preterm birth seems clear, the mechanism or mechanisms underlying this relationship have yet to be defined.

The increased risk of smoking-induced preterm birth is usually linked to pregnancy complications that include abruptio placentae, placenta previa, gestational bleeding, and PROM (4, 12). In addition, CS (or its individual constituents) may cause preterm delivery through impairment of placental function. For example, carbon monoxide and nicotine have been shown to reduce maternal blood supply to the placenta and, thus, to reduce oxygen supply to the fetus (13, 14).

Another mechanism by which CS may be related to preterm delivery is via the alteration of hormonal secretion patterns that are important for gestation, parturition, or both. The exact roles that progesterone, estrogen, and relaxin play in these processes vary among species. In general, progesterone maintains uterine quiescence during pregnancy (15). In most species, peripheral progesterone concentration declines before the initiation of labor (16, 17); in humans, progesterone is functionally withdrawn through a reduction in receptor expression before parturition (18). This withdrawal removes the suppression of estrogen receptor–α expression (19) and permits estrogen activation of contractile pathways including increased expression of contractility-associated genes (i.e., cyclo-oxygenase type 2, connexin-43, and the oxytocin receptor) and myometrial responsiveness to uterotonins (15). Along with estrogen, relaxin also aids in parturition by increasing the flexibility of the pubic symphysis and pelvic girdle, as well as the dilatability of the uterine cervix (20, 21).

Although information concerning the relationship among smoking during pregnancy, altered hormone levels, and preterm birth is limited, pervious studies have demonstrated that unfractionated CS can act as an antiestrogen by affecting the 2-hydroxylation pathway of estradiol metabolism leading to increased production of 2-hydroxyestrogens, which have minimal estrogenic activity and are rapidly cleared from the circulation (22, 23). Further support for its antiestrogenic effects comes from an *in vitro* study investigating the effects of CS alkaloids on progesterone biosynthesis. In this study, incubation of MA-10 cells (a gonadotropin-responsive, progesterone-synthesizing clonal strain) with either nicotine, cotinine (a nicotine metabolite), anabasine (an aromatase inhibitor), a mixture of these alkaloids, or an aqueous extract of CS resulted in a dose-dependent inhibition of progesterone synthesis (24).

Effects of CS exposure on prolactin are still being debated. Acute CS exposure has been reported to increase plasma levels of prolactin, while other investigations have demonstrated that baseline prolactin levels in chronic female smokers were significantly below those of nonsmokers (25, 26). Moreover, mothers who smoked a minimum of five cigarettes/day had lower serum levels of prolactin at the end of pregnancy (35–38 weeks) than nonsmoking mothers (27); studies in rats exposed to CS during pregnancy have also shown reduced (compared with the air-exposed controls) serum prolactin concentrations at the end of gestation (28).

The current study investigated the effects of gestational mainstream CS (MCS) exposure on preterm birth using a mouse model. This animal model was selected specifically for study because it has built-in and readily measurable "indicators" of impending parturition, including growth of an interpubic ligament and changes in the progesterone to estrogen ratio. A number of gestational parameters that signal approaching parturition, including interpubic ligament formation and hormone secretion patterns, were therefore investigated to better understand the mechanism or mechanisms by which exposure of pregnant dams to a dose of MCS equivalent to smoking less than one pack of cigarettes/day could lead to early delivery.

Materials and Methods

Animals. B6C3F1 male and female mice purchased from Jackson Laboratory (Bar Harbor, ME) were between 9 and 11 weeks of age at the time of arrival. Female mice were housed in pairs; males were housed individually in polycarbonate cages with corncob bedding in rooms with controlled temperature (20°–23°C) and humidity (~55% relative humidity). Mice were provided food (Purina 5001 lab chow) and tap water *ad libitum*. The light/dark cycle was maintained on a 12-hr interval. Mice were acclimated for at least 1 week before they were used in any experiments. All animal procedures were conducted under an animal protocol approved by New York University Institutional Animal Care and Use Committee.

Experimental Design. Sixty-eight female mice (10–12 weeks of age and weighing $\geq$20 g) were used for mating; two females were paired for 2 days with a single male (the first day of pairing was considered as Gestational Day 0 (GD 0). After pairing, the males were removed and the female mice were randomly assigned to one of two groups (A or B). Half of the dams (housed two per cage) from each group were exposed via whole-body inhalation (4 hrs/day for 5 days/week) to MCS and the remaining half were exposed for the same duration to filtered air. As shown in Figure 1, weighed dams from Group A (eight dams/exposure group) were exposed to either MCS or filtered air from GD 2 until sacrifice on GD 18. At the time of sacrifice, blood was collected from each dam and the serum was frozen (–70°C) until it was used to measure pregnancy hormone levels as described below. Fetal number (*in utero* litter size) and interpubic ligament length were also

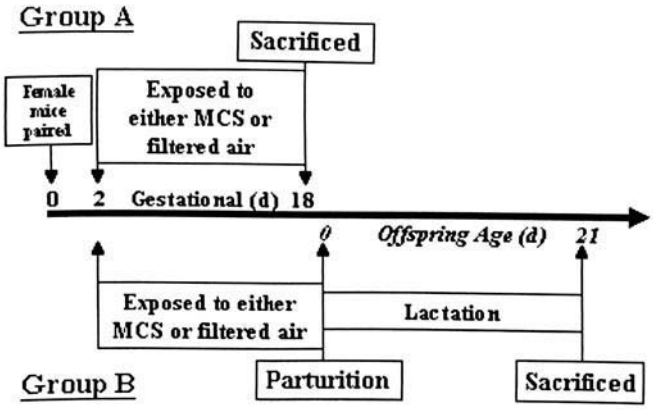

Figure 1. After pairing for 2 days, female mice were randomly assigned to one of two groups (A or B); half of the dams from each group were exposed to MCS and the remaining half to filtered air. Dams from Group A were exposed from GD 2 until sacrifice on GD 18; or, exposed until parturition and allowed to give birth (Group B).

determined for each dam. The pubic symphysis was exposed by dissection and the interpubic ligament length was measured at ×10 magnification using a dissecting microscope fitted with an ocular micrometer and a trans-illuminating device (29). Dams from Group B (9–10 dams/exposure group), exposed to either MCS or air from GD 2 until parturition, were allowed to give birth, and gestational parameters including duration of gestation and litter size (number of viable pups/litter), were determined. Following parturition, all mother/offspring sets were maintained in clean, filtered air. After nursing for 3 weeks, pups were separated from their mothers, the offspring sex ratio (female to male ratio) was assessed, and the dams were sacrificed for examination of postimplantation loss ([number of implantation sites – number of viable pups]/number of implantation sites).

Smoke Generation and Exposure. Half of the dams from each group were exposed via whole-body inhalation to either MCS or filtered air. MCS was generated from the burning of filtered 1R3F cigarettes (Kentucky Tobacco Research & Development Center, Lexington, KY). Smoke generation and animal exposure were performed as described previously by this laboratory (30). Chamber levels of carbon monoxide and total suspended particulates (TSP) were monitored throughout the exposure and averaged to 21.5 (± 0.5 [SE]) parts per million and 16.2 (± 0.9 [SE]) mg/m^3, respectively. Under these exposure conditions, levels of blood cotinine and carboxyhemoglobin (COHb), measured in three to four smoking dams on GD 18 averaged 28.1 (± 1.7 [SE]) ng/ml and 3.6% (± 0.1% [SE]), respectively (30); cotinine and COHb levels were below assay detection limits of 10 ng/ml and 2.4% (± 0.2%), respectively, in the air-exposed dams.

Hormone Measurements. Blood collected from Group A female mice exposed to either MCS or filtered air was used to determine maternal hormone levels. Estradiol-17β and progesterone were measured by specific radioimmunoassays (RIAs) that included estradiol-17β (kit No. 07-138015) and progesterone (kit No. 07–17015) (ICN, Costa Mesa, CA), respectively. Relaxin was measured by a specific rat relaxin RIA (31) and reagents provided by the innovator, Dr. O.D. Sherwood. Relaxin in mice cross-reacts with antisera to rat relaxin; dilutions of mouse sera were parallel to standards of rat relaxin. Prolactin was assayed using a specific rat prolactin RIA provided by Dr. A.F. Parlow (Scientific Director of the National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program). Dilutions of mouse sera were parallel to rat relaxin standards.

Statistical Analysis. Steroid determinations were conducted in duplicate, and prolactin and relaxin assays were run in triplicate. One-way ANOVA was used to determine CS-induced effects on body weight gain, hormone levels, interpubic ligament lengths, litter size, duration of gestation, and sex ratio. Fisher's protected least significant difference analysis was used for *post hoc* testing when appropriate (Abacus Concepts, Inc., Berkeley, CA). Differences were considered significant when probability (P) values were < 0.05.

Results

Gestational Parameters. As shown in Table 1, *in utero* litter size (determined on GD 18) was similar for both the MCS- and filtered air–exposed females in Group A. However, examination on the same gestational day revealed a significant ($P < 0.05$) increase in interpubic ligament length in the smoke-exposed dams compared with their air-exposed counterparts. In addition, exposure to MCS from the second day of gestation to parturition significantly ($P < 0.05$) reduced duration of gestation by 3.4% in the Group B MCS-exposed females (compared with the air-exposed dams); alternatively, exposure to MCS had no effect on litter size, offspring sex ratio, or postimplantation loss.

Body weight gain of the dams, determined either from GD 2 to GD 18 (Group A) or from GD 2 to parturition (Group B), was unaffected by MCS exposure. Both the smoke- and air-exposed dams gained ~1.1–1.2 g/day over the course of pregnancy.

Hormonal Patterns. Inhalation of MCS significantly ($P < 0.05$) increased serum estradiol-17β levels in the smoke-exposed dams on GD 18 (Fig. 2A), while progesterone levels in the same group were only slightly lower than those measured in the air-exposed females (Fig. 2B). The ratio of progesterone to estradiol-17β on GD 18 was significantly ($P < 0.05$) reduced in MCS-exposed pregnant dams (Fig. 2C), whereas prolactin levels tended ($P = 0.07$) to be higher than control values (Fig. 2D). Relaxin levels were unaltered by MCS exposure and averaged 1.3 (± 0.2 [SE]) ng/ml and 1.4 (± 0.1) ng/ml in the air- and smoke-exposed dams ($n = 8$ dams/exposure group), respectively.

Table 1. Effects of Mainstream Cigarette Smoke Exposure on Gestational Parameters

Group[a]	Gestational parameters	Exposure group[b]	
		Filtered air	Cigarette smoke
A	Litter size (no. of pups *in utero*/litter)	8.8 ± 0.5	8.6 ± 0.4
	Interpubic ligament length (mm)	2.5 ± 0.1	3.4 ± 0.1*
B	Litter size (no. of viable pups/litter)	7.8 ± 0.8	7.8 ± 0.5
	Duration of gestation (days)[c]	20.3 ± 0.2	19.6 ± 0.2*
	Female to male sex ratio	1.0 ± 0.2	1.4 ± 0.5
	Postimplantation loss ([no. of implantation sites − no. of viable pups]/no. of implantation sites)	0.06 ± 0.03	0.04 ± 0.02

[a] Parameters evaluated for Groups A and B were determined on GD 18 and after parturition, respectively.
[b] Values represent the mean (Group A; $n = 8$ dams/exposure group; Group B; $n = 9$–10 dams/exposure group) ± SE.
[c] The first day of pairing was considered as GD 0; mice were paired for 2 days.
*Significantly different ($P < 0.05$) from air-exposed controls.

Discussion

The present study reports on the development of a pregnant mouse model to study the effects of MCS on serum hormone patterns and on some important preparturient changes in the reproductive tract in relation to preterm delivery. Pregnant mice exposed to a CS particle and carbon monoxide concentration roughly equivalent to smoking less than one pack of cigarettes per day demonstrated no overt signs of toxicity such as impaired maternal weight gain or decreased litter size, as has been reported for rodents exposed to higher CS concentrations (32, 33). However, in the absence of any obvious toxicologic effects, length of gestation was decreased by 3.4%. Although this may seem as only a modest reduction in the mouse model, such a decrease in gestational duration is equivalent to a 1.3-week reduction in human pregnancy, which places the human fetus in the preterm delivery category (less than 37 weeks) (34, 35) and, thus, at increased risk for a variety of well-established adverse health outcomes.

Mice have a built-in "indicator" of approaching parturition; namely, the development of a long ligament between the pubic bones, accompanied by increased flexibility of the entire pelvic girdle; without these changes in flexibility, parturition may fail (36–38). Commensurate with impending preterm delivery, the pubic joints of the CS-exposed mice exhibited significantly longer interpubic

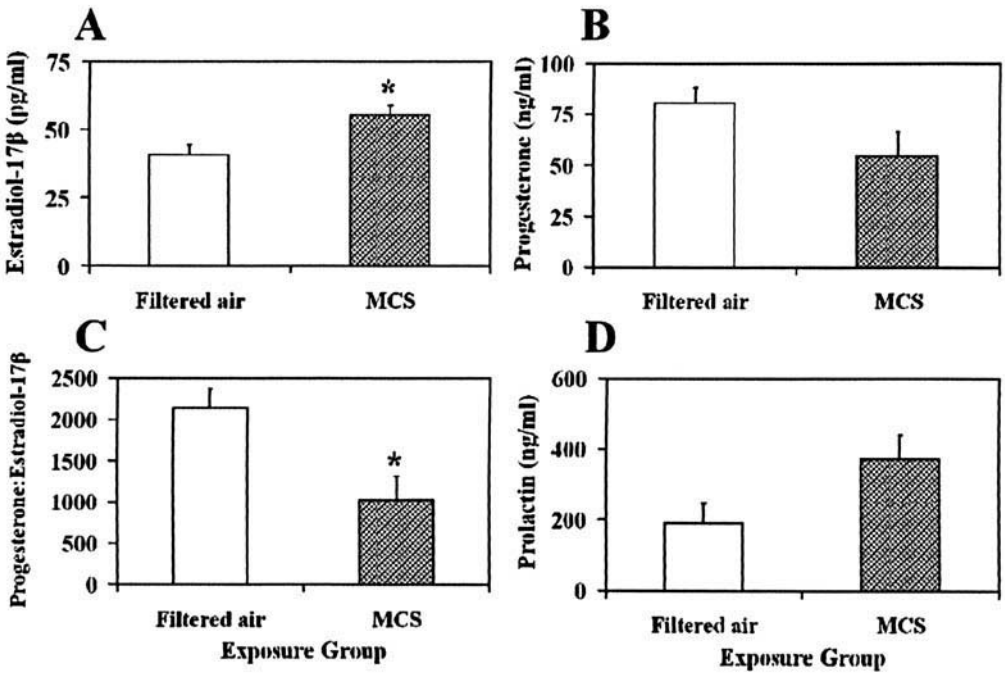

Figure 2. Effects of inhaled MCS on serum (A) estradiol-17β ($n = 7$–8 dams/exposure group), (B) progesterone ($n = 6$ dams/exposure group), (C) progesterone to estradiol-17β ratio ($n = 6$ dams/exposure group), and (D) prolactin ($n = 7$ dams/exposure group) in pregnant female mice determined on GD 18. Values represent the mean ± SE. *Significantly different ($P < 0.05$) from air-exposed dams.

ligaments on GD 18 compared with those of the filtered air controls measured on the same gestational day.

Interpubic ligaments grow in preparation for parturition under the stimulus of increasing levels of estradiol and relaxin (37, 39). However, this process also depends upon a decrease in serum progesterone, which antagonizes the effects of estradiol and relaxin on the pubic symphyseal connective tissue during pregnancy. As the time of delivery approaches, progesterone secretion declines allowing estradiol and relaxin to induce growth of the interpubic ligament (38). Therefore, the observed increase in interpubic ligament length in the CS-exposed mice accompanied by significant increases in serum estradiol and decreases in the progesterone to estradiol ratio (relative to the filtered air controls) provides a mechanism whereby CS induces preterm delivery.

Although it is well established that smoking during pregnancy increases the risk of preterm delivery, the mechanisms responsible for this phenomenon are poorly understood (40). A number of studies have indicated that maternal hormones such as progesterone play an important role in CS-associated preterm birth. Certain substances in CS, notably cadmium and nicotine, have been shown *in vitro* to inhibit the synthesis or release (or both) of progesterone from human granulosa cells (41) and luteal cells (42), as well as from cultured trophoblasts (43). In another study, cultured rat ovary and placenta treated with the smoke constituent cadmium produced less progesterone than the nontreated control tissues; human placental tissues recovered from smoking mothers also produced less progesterone than those tissues taken from their nonsmoking counterparts (44). Likewise, cadmium has been shown to inhibit progesterone synthesis *in vivo* and also to bind to estrogen receptors *in vitro* (45). In pregnant rats, inhaled CS enhanced the *in vitro* response of uterine segments to oxytocin, and induced a significant increase in expression of oxytocin-receptor messenger RNA (40). Thus, smoke constituents acting as endocrine disruptors may play a role in inducing preterm births in our mouse model.

Clinical studies support an important role for progesterone insufficiency in the etiology of early delivery. Treatment with a naturally occurring progesterone metabolite (i.e., 17-hydroxyprogesterone as the caproate ester) significantly decreased the incidence of early deliveries in women with a history of such events (46, 47). However, meta-analyses of several studies in which progesterone itself was administered to women with a history of preterm births have yielded variable data (48–50). While few animal models of preterm birth have been reported, pregnant rabbits treated with the progesterone antagonist RU486 delivered prematurely and exhibited significantly reduced serum and uterine progesterone concentrations in comparison with the untreated controls (51).

Relaxin, a luteal hormone, has been shown in animal studies to also be involved in many aspects of parturition including uterine contractility and oxytocin response, along with preparation of the cervix and pelvic ligaments for the birthing process (20, 39, 52). Elevated serum relaxin levels observed in women during the first trimester are maintained throughout pregnancy and are linearly related to preterm birth (53). Moreover, expression of relaxin genes and proteins in the human deciduas and placenta is increased in patients with preterm premature rupture of the fetal membranes (54). In the present study, serum relaxin concentration appeared to be unaffected by MCS exposure. However, the observed increase in estradiol coupled with the decrease in the progesterone to estradiol ratio might have led to an enhanced response of the uterus to relaxin, as well as to a net increase in interpubic ligament length in the CS-exposed dams, clearly signaling approaching parturition.

These toxicologic studies have demonstrated that exposure of pregnant dams to even modest MCS concentrations can alter pregnancy hormone levels that can accelerate changes in the reproductive tract necessary for parturition. Furthermore, these studies also show that such hormonal alterations may be at least partly responsible for preterm deliveries associated with CS exposure. Another important outcome of this study was the development of an animal model that could demonstrate CS-induced changes in gestation and parturition in a short time span, as well as accurately reflect the changes in hormone levels and reproductive tract connective tissues occurring over weeks or months in the human counterpart. Such a model may be extremely useful for estimating human risk, as well as for evaluating recently developed "smarter" or "safer" cigarettes.

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