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An animal model of cigarette smoke-induced *in utero* growth retardation

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Abstract

Maternal/fetal genetic constitution and environmental factors are vital to delivery of a healthy baby. In the United States (US), a low birth weight (LBW) baby is born every minute and a half. LBW, defined as weighing less than 5.5 lbs at birth, affects nearly 1 in 12 infants born in the US with resultant costs for the nation of more than 15 billion dollars annually. Infant birth weight is the single most important factor affecting neonatal mortality. Various environmental and genetic risk factors for LBW have been identified. Several risks are preventable, such as cigarette smoking during pregnancy. Over one million babies are exposed prenatally to cigarette smoke accounting for over 20% of the LBW incidence in the US. Cigarette smoke exposure *in utero* results in a variety of adverse developmental outcomes with intrauterine growth restriction and infant LBW being the most well documented. However, the mechanisms underlying the causes of LBW remain poorly understood. The purpose of this study was: (1) to establish an animal model of cigarette smoke-induced *in utero* growth retardation and LBW using physiologically relevant inhalation exposure conditions which simulate “active” and “passive” tobacco smoke exposures, and (2) to determine whether particular stages of development are more susceptible than others to the adverse effects of *in utero* smoke exposure on embryo/fetal growth. Pregnant C57BL/6J mice were exposed to cigarette smoke during three periods of gestation: pre-/peri-implantation (gestational days [gds] 1–5), post-implantation (gds 6–18), and throughout gestation (gds 1–17). Reproductive and fetal outcomes were assessed on gd 18.5. Exposure of dams to mainstream/sidestream cigarette smoke, simulating “active” maternal smoking, resulted in decreases in fetal weight and crown–rump length when exposed throughout gestation (gds 1–17). Similar results were seen when dams were exposed only during the first 5 days of gestation (pre-/peri-implantation period gds 1–5). Exposure of dams from the post-implantation period through gestation (gds 6–18) did not result in reduced fetal weight, although a significant reduction in crown–rump length remained evident. Interestingly, maternal sidestream smoke exposure, simulating exposure to environmental tobacco smoke (ETS), during the pre-/peri-implantation period of development also produced significant decreases in fetal weight and crown–rump length. Collectively, results from the present study confirm an association between prenatal exposure to either “active” or “passive” cigarette smoke and *in utero* growth retardation.

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The data also identify a period of susceptibility to *in utero* cigarette smoke exposure-induced growth retardation and LBW during pre-/peri-implantation embryonic development.

Keywords

Embryo; Fetus; Pre-implantation; Cigarette smoke; Tobacco; Low birth weight

1. Introduction

Development of a healthy, full-term baby during pregnancy is contingent on many factors including: maternal and fetal genetic makeup, maternal/fetal interactions, and environmental influences. Low birth weight (LBW) is an adverse developmental outcome, the real importance of which has only recently come to be appreciated. One out of every twelve infants born in the United States (US) have LBW, which is classified as weighing less than 5.5 lbs at birth (Das and Sysyn, 2004; Peristats, 2007). Two sources from which LBW can originate include: (1) preterm/premature births, defined as those occurring before the 37th week of gestation and also weighing less than 5.5 lbs at birth and (2) intrauterine growth retardation (IUGR), defined as a process that contributes to a reduction in fetal growth potential consequently causing a smaller than expected infant. In addition, small for gestational age (SGA) is a term used to describe growth restricted infants, but are strictly classified as those who weight less than three percent of mean birth weight standards (weighing two standard deviations below the mean) for a given gestational age (Das and Sysyn, 2004). Consequently, LBW is one of the most prevalent factors involved in neonatal mortality contributing to over 65% of infant deaths (Das and Sysyn, 2004; Centers for Disease Control and Prevention, 2006a; Peristats, 2007). Moreover, LBW infants surviving past their first year of life face an increased risk of long-term disabilities such as cognitive deficits, obesity, hypertension, cardiovascular disease, and diabetes (Botero and Lifshitz, 1999; Ong and Dunger, 2002; Das and Sysyn, 2004; Gluckman et al., 2005). While a number of genetic and environmental risk factors for LBW have been identified, one of the best-documented environmental factors contributing to LBW is maternal smoking status (Comstock et al., 1971; Magee et al., 2004; Bailey and Byrom, 2007).

Despite increased public awareness regarding the adverse health effects of smoking, tobacco use remains prevalent in the home, public facilities, and the workplace. In the United States, the percent of adult cigarette smokers is 20.2% when classified as persons smoking greater than 100 cigarettes in a lifetime and currently smoking everyday. That percentage increases to 25.3% for persons of childbearing ages (18–35 years) (Centers for Disease Control and Prevention, 2007). Although the numbers vary between race/ethnic backgrounds, approximately one in five women will smoke sometime during her pregnancy (Centers for Disease Control and Prevention, 1998, 2001). Statistics correlating maternal smoking and LBW follow similar trends, with smoking accounting for over 20% of LBW incidences (Centers for Disease Control and Prevention, 2001). In the United States alone, approximately one million infants are exposed *prenatally* to cigarette smoke each year (Centers for Disease Control and Prevention, 1998). *In utero* smoke exposure has been shown to result in a variety of adverse developmental outcomes, with infant LBW being the most well documented in humans (Abel, 1980; Hebel et al., 1988; Cooke, 1998; Bernstein et al., 2000; Mitchell et al., 2002). In addition to LBW, maternal smoking status has been linked to a variety of other adverse pregnancy outcomes including: placental insufficiency, placental abruption, sudden infant death syndrome (SIDS), childhood asthma/bronchitis, and cognitive and learning deficits (Klein and Koren, 1999; Lindley et al., 2000; Ernst et al., 2001; Cnattingius, 2004; Lahr et al., 2005). While exposure to environmental tobacco smoke (ETS, also known as “passive” or second-hand smoke) has been considered as a “serious health hazard” (Centers for Disease Control and Prevention, 2006b), associations between ETS exposure during

pregnancy and adverse pregnancy outcomes are still controversial (Witschi et al., 1995; Windham et al., 1999; Kharrazi et al., 2004).

Animal models have been used to examine the effects of tobacco smoke on pre- and post-natal developmental outcomes (Gaworski et al., 2004; Pauly et al., 2004). In many of these studies however, pregnant animals were only exposed to nicotine, under the assumption that nicotine is the *principal* developmental toxicant in cigarette smoke (Navarro et al., 1989; Roy et al., 1998; Pauly et al., 2004). Results from other studies in which pregnant rodents were exposed to cigarette smoke have been equivocal, due no doubt to differences in exposure conditions, dosing times and concentrations of total suspended particulates (Reznik and Marquard, 1980; Seller and Bnait, 1995; Carmines et al., 2003). Interpretation of such studies is further complicated by the fact that cigarette smoke is comprised of over 4000 identifiable compounds, and approximately 600 additives (National Cancer Institute, 1999). Moreover, two types of cigarette smoke each containing distinct chemical profiles have been identified: mainstream smoke and sidestream smoke (Schick and Glantz, 2005). Mainstream smoke, simulating “active” smoking, is defined as that derived from the puff end of the cigarette. In contrast, sidestream smoke is defined as that generated from both the smoldering end of a cigarette, between puffs, as well as the smoke exhaled by a smoker. Inhalation of sidestream smoke is said to result in environmental, second-hand, or “passive” tobacco smoke exposure (National Cancer Institute, 1999).

The purpose of the current study was: (1) to establish an animal model of *in utero* cigarette smoke exposure-induced growth retardation and low birth weight using physiologically relevant routes of smoke exposure which simulate “active” as well as “passive” tobacco smoke exposures, and (2) to determine whether particular stages of development are more susceptible than others to the adverse effects of *in utero* smoke exposure on embryo/fetal growth.

2. Materials and methods

2.1. Materials

The Teague TE-10C cigarette smoke inhalation exposure system was purchased from Teague Enterprises (Davis, CA). Marlboro Red Class 20 A cigarettes were obtained from a local commercial vendor. Unless otherwise designated, all chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Methanol was purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Cigarette smoke inhalation exposure system

The Teague TE-10C, previously described in detail (Teague et al., 1994), is a microprocessor controlled cigarette-smoking apparatus that delivers cigarette smoke to an enclosed inhalation chamber. Both inhalation chambers (sham/ambient air and cigarette smoke exposed) are each 28” in length by 24” in height by 24” in width. This instrument can produce sidestream smoke, mainstream smoke, or a combination of both. Sidestream/mainstream combination smoke, simulating “active smoking”, was generated using Marlboro Red Class 20 A cigarettes. Sidestream cigarette smoke was used to simulate environmental tobacco smoke (ETS) or “passive” smoke exposure.

Commercially available cigarettes were chosen over Reference cigarettes since the later do *not* contain the 600 potential harmful additives, included in commercially available cigarettes (National Cancer Institute, 1999), nor are they commonly smoked by the general public. While there are differences in cigarette preference among racial/ethnic groupings, Marlboro cigarettes are the most popular brand of cigarettes among young adults (aged 18–25), with women of this age representing the majority of maternal smokers (Substance Abuse and Mental Health Services Administration, 1999).

Cigarettes were stored at 4 °C until 48 h prior to use when they were placed into a closed chamber at 23 °C containing a glycerin/water mixture to establish a relative humidity of 60%. Cigarettes were smoked using the standard Federal Trade Commission (FTC) method: a two second, 35 cm³ puff, once a minute for a total of nine puffs (Teague et al., 1994). Sidestream smoke, generated from the smoldering end of the cigarette between puffs, was collected in the chimney area above the cigarette carrier and drawn into a conditioning chamber. Mainstream smoke was collected from the puff port and was also directed into the conditioning chamber. Collected smoke was then diluted with fresh air and passed into an exposure chamber where pregnant mice were placed during the treatment period.

Total suspended particulates (TSPs), temperature, carbon monoxide (CO) levels, and humidity of the exposure chamber were determined twice during each daily exposure session. TSPs were collected using 25 mm Teflon-coated filters (TX40H120-WW, Pallflex Products Co.; Putnum, CT). Chamber temperature and humidity were monitored with a digital hygrometer/thermometer (Control Company; Friendsworth, TX) and carbon monoxide levels were measured with a digital display carbon monoxide detector (KIDDE; Mebane, NC).

2.3. Animals

C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME). All animals were housed in ventilated-racks in a room dedicated to the smoke inhalation exposure system and were maintained by the University of Louisville Research Resources Center, an AAALAC-accredited facility. Animals were kept in a room with a 12-h light/dark cycle at 22 °C with *ad libitum* access to food and water. Timed pregnancies were established by overnight mating of a single mature male with two nulliparous females. The presence of a vaginal plug was considered evidence of mating and the time considered day 0 of gestation (gd 0). Pregnant mice were exposed to cigarette smoke or ambient air (sham-exposed control) for 6 h per day over three different developmental time periods: pre-/peri-implantation period of gestation (gds 1–5), post-implantation period of gestation (gds 6–18), throughout gestation (gds 1–17) (Fig. 1). Dams were weighed daily during the smoke exposure period and monitored for signs of toxicity: weight loss, moribundity, mortality, ruffled fur, reluctance to ambulate, inappetence, and chromodacryorrhea. In order to limit any stress associated with solitary housing, every attempt has been made to keep animals group-housed during both the exposure periods as well as the time between treatments. Pregnant mice were excluded from embryo outcome and dam weight gain assessments if the dam was determined not to be pregnant or had less than four implantations.

2.4. Animal inhalation exposures

On the morning of the daily experimental smoke exposure, dams were weighed and exposed to either ambient air (sham) or cigarette smoke [cigarette smoke-exposed (CSE)] in paired inhalation chambers during the gestational intervals specified above for each experiment (see Fig. 1 for overview). Sham and CSE dams were exposed for 6 h/day. Dam tail blood was collected immediately after the smoke exposure session, on designated days of gestation, as noted below.

2.5. Cotinine determination as a biomarker of cigarette smoke exposure

Levels of cotinine, the principal metabolite of nicotine, and a well-documented marker of “active” and “passive” tobacco smoke exposure (Society for Research on Nicotine, Tobacco Subcommittee on Biochemical Verification, 2002), were monitored in the sham-exposed and CSE dams. At designated time points during the exposure period (gd 3 for the pre-/peri-implantation study, and gd 6 for the post-implantation and throughout gestation studies) tail blood was collected from the dams into heparinized capillary tubes. Blood samples were centrifuged at 10,000 × *g* for 10 min and plasma fractions were collected and stored at –20 °

C until analyzed. Plasma was assayed for cotinine concentrations using the Cotinine One-Step ELISA Detection Kit (International Diagnostic Systems; St. Joseph, MI). This procedure uses a competitive binding assay in which plasma cotinine competes with a cotinine–enzyme–conjugate for binding to a cotinine-specific antibody. The amount of bound conjugate is then detected by measuring absorbance at 450 nm of the substrate tetramethylbenzidine (TMB). Cotinine concentrations were determined against a standard curve (0–50 ng/ml) and the data are reported as mean ng/ml cotinine $\pm$ standard error of the mean (S.E.M.).

2.6. Outcome measures/“fetal phenotyping”

On gd 18.5, dams were anesthetized with Avertin (500 mg/kg, i.p.) (Nagy et al., 2003) and then euthanized by carbon dioxide asphyxiation followed by cervical dislocation. The uterine horns were exteriorized, the number and location of implantations and resorptions were recorded, and the fetuses were removed from the uterus, blotted, weighed, measured for crown–rump length, and examined for gross external abnormalities.

2.7. Statistical analyses

Data was analyzed with the Statistical Package for Social Sciences[®] (SPSS; Chicago, IL) version 13.0. Since the dam was the treated entity, the litter rather than the individual embryo/fetus was regarded as the experimental unit (Haseman and Hogan, 1975). Data on treatment-induced changes in dam weight gain were analyzed using an independent samples *t*-test. The statistical analysis on fetal outcomes data was conducted using a multivariate ANOVA, with the fixed factor being treatment and the number of implantations, number of resorptions, number of viable fetuses, fetal weight, fetal crown–rump length, fetal brain weight, and number of abnormalities as the dependent variables. Pearson's correlation analysis (number of viable fetuses vs. fetal body weight) was performed on data from each experimental paradigm to address whether litter size correlates to fetal weight in our model. Data are presented as means $\pm$ standard error of mean (S.E.M.). Statistical significance was assigned $p < 0.05$.

3. Results

3.1. Mainstream/sidestream cigarette smoke exposure

3.1.1. Chamber conditions—Throughout all experimental paradigms, inhalation exposure chamber conditions, such as carbon monoxide levels, relative humidity, temperature and TSP, were monitored during the exposure periods. Table 1 reports the average chamber conditions for all mainstream/sidestream cigarette smoke exposures during the three gestational periods (gds 6–18, gds 1–17, and gds 1–5) examined. Mean CO levels and total suspended particulates (TSP) in the cigarette-smoke exposed chamber were 128.0 ± 3.0 ppm and 29.2 ± 2.6 mg/m³, respectively. TSP levels are used to represent the “dose” of mainstream/sidestream cigarette smoke to which the animal was exposed, while plasma cotinine levels are a physiological indicator of the actual smoke exposure of the animal. In the current study, mainstream/sidestream cigarette smoke exposure of the dams, achieving TSP levels in the range noted above (29.2 ± 2.6 mg/m³), resulted in average dam plasma cotinine levels of 96.4 ± 18.6 ng/ml. Since, the minimum accepted criterion for classification of “active” smoking is a plasma cotinine level of ≥ 50 ng/ml (Koren et al., 1992; Society for Research on Nicotine, Tobacco Subcommittee on Biochemical Verification, 2002), and since cotinine values in dams undergoing the current mainstream/sidestream smoke inhalation exposure paradigm achieved/exceeded this set criterion, this particular model achieves an average level of smoke exposure that simulates that of “active” maternal smoking during pregnancy.

3.1.2. Effects of mainstream/sidestream cigarette smoke exposure during the post-implantation period (gds 6–18)—Dams were exposed to Marlboro mainstream/sidestream cigarette smoke in the post-implantation period, commencing on gd 6 and

continuing through gd 18, as detailed in Section 2. This post-implantation period includes events such as organo-genesis and late term fetal growth. During the post-implantation period of development, chamber conditions remained consistent (Table 1). Reproductive and fetal outcomes following *in utero* smoke exposure on gds 6–18 are detailed in Table 2. Results indicated a significant effect of smoking on fetal crown–rump length (27.4 ± 0.3 mm) compared to sham-exposed controls (28.5 ± 0.3 mm). In contrast to our predicted hypothesis, exposure to cigarette smoke during the post-implantation period did not result in statistically significant alterations in fetal weight.

3.1.3. Effects of mainstream/sidestream cigarette smoke exposure throughout gestation (gds 1–17)—As *in utero* exposure to cigarette smoke in the post-implantation period did not affect fetal weight, it was decided that the effects of *in utero* cigarette smoke exposure *throughout* gestation would be examined. Towards this end, dams were exposed to Marlboro mainstream/sidestream cigarette smoke, commencing on gd 1 and continuing through gd 17, as detailed in Section 2. Inhalation exposure chamber conditions for the study conducted throughout gestation were comparable to those for the study conducted during the post-implantation period (Table 1). As delineated in Table 3, smoke exposure of the dams throughout gestation resulted in a statistically significant decrease in *three* fetal outcomes: fetal weight (1.15 ± 0.03 g [sham] vs. 1.08 ± 0.02 g [CSC]) (see also Fig. 2A), crown–rump length (28.4 ± 0.3 mm [sham] vs. 27.1 ± 0.2 mm [CSE]) (see also Fig. 2B), and brain weight (80.6 ± 1.1 mg [sham] vs. 75.6 ± 1.3 mg [CSE]). Fig. 2C and D illustrate representative fetuses (sham-exposed fetus Fig. 2C and a smoke-exposed fetus Fig. 2D) on gestational day 18.5, reflecting the 6% reduction in weight observed following cigarette smoke exposure on gestational days 1–17. These data (Tables 1 and 2, Fig. 2) collectively substantiate the successful establishment of an *in utero* cigarette smoke exposure-induced low birth weight animal model and suggest a temporal window of vulnerability during development for this effect.

3.1.4. Effects of mainstream/sidestream cigarette smoke exposure during the pre-/peri-implantation period (gds 1–5)—In order to determine whether the pre-/peri-implantation period [gds 1–5 in the mouse] (Surani, 1975; Card and Mitchell, 1979; Kaufman, 1992), represents a uniquely vulnerable developmental interval for *in utero* smoke exposure-induced low birth weight, pregnant dams were exposed to mainstream/sidestream cigarette smoke on days 1–5 of gestation as detailed in Section 2. Inhalation exposure chamber conditions for the study conducted during the pre-/peri-implantation period of development were comparable (Table 1) to those in the other two above-noted experimental treatment paradigms. Exposure of the dams to cigarette smoke only during days 1–5 of gestation resulted in statistically significant decreases, at gestational term (gd 18.5), in fetal weight (1.18 ± 0.02 g [sham] vs. 1.10 ± 0.01 g [CSE]) and crown–rump length (28.7 ± 0.2 mm [sham] vs. 27.4 ± 0.2 mm [CSE]) (Table 4; Fig. 3A and B). Collectively, these data identify the pre-/peri-implantation period of development as a critical window of vulnerability to *in utero* cigarette smoke exposure-induced decreases in fetal weight during gestation.

3.2. Sidestream cigarette smoke exposure

3.2.1. Effects of sidestream cigarette smoke exposure during the pre-/peri-implantation period (gds 1–5)—Given that embryonic development during the pre-/periimplantation period was sensitive to mainstream/sidestream cigarette smoke exposure and resulted in decreased fetal weight and crown–rump length, the effects – during this same developmental period – of *only sidestream* cigarette smoke exposure on fetal outcomes were determined. Exposure of dams to a treatment regimen of sidestream cigarette smoke, which simulated a human “second-hand smoke” exposure, resulted in lower chamber levels of CO (35.7 ± 0.8 ppm) and (5.99 ± 0.48 mg/m³) (Table 5), as well as average dam peak cotinine levels of 42.1 ± 7.7 ng/ml which are within the criteria set for environmental tobacco smoke

exposures (<50 ng/ml) (Koren et al., 1992; Society for Research on Nicotine, Tobacco Subcommittee on Biochemical Verification, 2002). Surprisingly, exposure to *only* the sidestream component of cigarette smoke during the pre-/peri-implantation period – equivalent to a human “passive exposure” – was sufficient to result in significant decreases in fetal weight (1.17 ± 0.01 g [sham] vs. 1.13 ± 0.01 g [CSE]), crown–rump length (28.4 ± 0.3 mm [sham] vs. 27.6 ± 0.2 mm [CSE]), and brain weight (79.0 ± 0.7 [sham] vs. 75.5 ± 1.1 mg [CSE]) (Table 6; Fig. 3C and D).

In the “passive” cigarette smoke exposure paradigm, delivery of sidestream smoke generated average TSP values in the inhalation chambers that were approximately one-fifth of those generated by delivery of mainstream/sidestream smoke (5.99 ± 0.48 mg/m³ vs. 29.2 ± 2.6 mg/m³), and resulted in dam plasma cotinine levels that were approximately two-fifths of the levels in mainstream/sidestream smoke-exposed dams. Despite decreased delivery of TSP to, and decreased plasma cotinine levels of, dams exposed to sidestream smoke during the pre-/peri-implantation period of development, a statistically significant reduction in fetal growth (weight and crown–rump length) was apparent. Moreover, when compared to the decrease in growth observed in the mainstream/sidestream smoke-exposed cohort, the magnitude of the decrease was less in the “passive” exposure cohort, suggesting a potential exposure (dose)-dependent effect. For example, while exposure to mainstream/sidestream cigarette smoke during gds 1–5 produced an overall decrease in fetal weight of approximately 6.8%, and a decrease in fetal crown–rump length of 4.5%, sidestream smoke exposure during this same developmental period produced an attenuated response—3.4% reduction in fetal weight and 2.8% decrease in fetal crown–rump length. Since TSP values and plasma cotinine levels are indicative of the amount of smoke delivered to the dam during the exposure period, these correlative fetal outcomes suggest a dose-dependent effect of cigarette smoke exposure on intrauterine growth.

4. Discussion

A number of studies using rodent animal models have been conducted to examine the effects of tobacco smoke on preand post-natal developmental outcomes (Gaworski et al., 2004; Pauly et al., 2004). However, in many studies, the experimental design involved exposure of the animals to nicotine, under the assumption that nicotine is the principal developmental toxicant in cigarette smoke (Navarro et al., 1989; Roy et al., 1998; Pauly et al., 2004). The Teague TE-10C cigarette smoke inhalation exposure system, used in the present study, has the capability of producing both mainstream and sidestream cigarette smoke from standard cigarettes. The utilization of an *inhalation exposure* system for delivery of smoke to pregnant mice, simulating human “active” (mainstream/sidestream combination) or “passive” (sidestream only) cigarette smoke exposure, is a more physiologically relevant model of human pregnancy exposure than exposure to individual cigarette smoke components, such as nicotine. While others have begun to utilize such systems to study the effects of tobacco smoke on pre- and post-natal development (Reznik and Marquard, 1980; Seller and Bnait, 1995; Carmines et al., 2003), the lack of consistency between studies (i.e. differences in dosing times, conditions, and animal models) makes comparisons difficult. The purpose of the current study was to (1) establish a viable animal model of smoking-induced low birth weight (LBW) using an inhalation route of exposure and (2) investigate potential developmental periods of sensitivity for cigarette smoke-induced LBW.

Collectively, data from the present study demonstrate that *in utero* exposure to cigarette smoke during the pre-/periimplantation period of murine gestation (gds 1–5) induces fetal growth inhibition and results in statistically significant decreased weight at term gestation. While *in utero* exposure to cigarette smoke during the post-implantation period of murine gestation (gds 6–18) resulted in decreased fetal crown–rump length, there was no statistically significant effect on fetal weight. It is well recognized that embryos are particularly vulnerable to toxic

insults during the early stages of organogenesis (Rice, 1976; Lambers and Clark, 1996). Moreover, studies have suggested that fetal weight may be principally regulated by placental growth function during mid-gestation (gds 11.5–13.5) (Ishikawa et al., 2006), suggesting that the maternal to fetal exchange of smoke constituents through the placenta during this time period would play a role in the generation of a LBW phenotype. Therefore, the lack of an effect on fetal weight, subsequent to *in utero* cigarette smoke exposure during days 6–18 of gestation, was quite unexpected.

Since initial data from the present study indicated that there was no statistically significant effect on fetal weight subsequent to *in utero* cigarette smoke exposure during gds 6–18, it was decided to expose dams throughout the entire gestational period. *In utero* cigarette smoke exposure commencing on gd 1 and continuing through gd 17 resulted in a statistically significant decrease in fetal weight at term. This finding cannot be attributed to altered maternal nutrition, as there was no significant difference in weight gain of the dams exposed throughout the *entire* gestational period. Moreover, the difference in fetal weight outcomes between the two exposure groups (pre-/periimplantation vs. throughout gestation) cannot be attributed to differences in the chamber environment, as TSP levels – indicative of the “dose” of smoke ($29.2 \pm 2.6 \text{ mg/m}^3$) and dam cotinine concentrations – indicative of *actual* smoke exposure ($96.4 \pm 18.6 \text{ ng/ml}$) were nearly identical (see standard error of the mean). Lastly, although our data demonstrated a significant negative correlation between litter size and fetal weight for three of the four data sets (data not shown), there are no significant treatment effects on litter size in the mainstream/sidestream gestational days 1–17 exposure or the sidestream gestational days 1–5 exposure, suggesting that this variable does not contribute to the observed reduction in fetal weight in this system. These data are in agreement with a number of studies suggesting that exposure to cigarette smoke throughout gestation reduces fetal weight (Leichter, 1989; Leichter, 1995; Seller and Bnait, 1995).

Collectively, data from the present study suggest that the pre-/peri-implantation stage of development may represent a period of distinct vulnerability to cigarette smoke-induced intrauterine growth retardation. Indeed, exposure of dams to cigarette smoke *only* during days 1–5 of gestation (the pre-/peri-implantation period) resulted in a statistically significant decrease in fetal weight (6.8%) that was comparable to the reduction in fetal weight obtained following smoke exposure of the dams throughout gestation (gds 1–17) (6.1%). Thus, *in utero* smoke exposure-induced effects on fetal weight are not exacerbated by smoke exposure continuing past day 5 of gestation. Moreover, exposure to *only* sidestream smoke (as opposed to combination sidestream/mainstream smoke) during gds 1–5 *also* resulted in a decrease in fetal weight (3.4%). Thus, both mainstream, as well as sidestream cigarette smoke should be considered as potentially harmful to the developing embryo during the pre-/peri-implantation period of development.

Cellular or molecular mechanisms responsible for *in utero* pre-/peri-implantation smoke exposure-induced fetal growth retardation are currently unknown. During this early period of gestation, embryonic cells are highly proliferative, segregate and polarize to form the two primary cell populations of the embryo and the placenta, and the cells forming the embryo begin to undergo a myriad of processes associated with implantation (Kaufman, 1992). In view of the fact that nicotine, the principal component of cigarette smoke, has been found to delay embryonic implantation, (Card and Mitchell, 1979) it is possible that smoke exposure-induced decreases in fetal weight are due to delayed embryo implantation and attendant generalized developmental delays. Although it has been suggested that maternal smoking-induced reductions in fetal weight are due to altered placental function and decreased maternal to fetal nutrient and oxygen exchange (Ashfaq et al., 2003; Mayhew et al., 2003; Myren et al., 2007), in the pre-/peri-implantation exposure model wherein dams are exposed to cigarette smoke from days 1 to 5 of gestation, the maternal/fetal placental blood exchange is not functional.

The development of the fully formed chorioallantoic placenta with maternal/fetal blood exchange and large blood-filled sinuses is not completed until gd 9.75 (Iwaki et al., 2002). Still, it is important to consider that the groundwork for development of the placenta itself (Molteni, 1984) is established during this window of susceptibility (gds 1–5). Hence, it cannot be ruled out that patterns and/or rates of fetal and/or placental growth may be determined during this temporal window.

In contrast to the distinct developmental period of vulnerability to smoke exposure-induced alterations in fetal weight gain, reductions in crown–rump length were uniformly observed in each of the exposure paradigms utilized in the present study—regardless of the exposure period (pre-/peri-implantation gds 1–5, post-implantation gds 6–18 or throughout gestation gds 1–17) or the type of cigarette smoke (mainstream/sidestream vs. sidestream only) to which the dams were exposed. These data are consistent with human studies showing that maternal smoking during pregnancy differentially alters the growth trajectory for weight vs. length of the fetus (Lindley et al., 2000). Effects on crown–rump length may, in part, be due to altered maternal nutrition as there was a decrease in dam weight gain in the cigarette smoke exposed group (11.8 ± 1.0 g) compared to the sham control group (14.4 ± 0.5 g) in the *post-implantation* (gds 6–18) cohort. However, there were no significant differences in dam weight gain between the two treatment groups exposed during gds 1–17 and gds 1–5, suggesting that maternal nutrition may not contribute to the consistent decreases in crown–rump length in the smoke-exposed fetuses. While it might be hypothesized that *in utero* smoke exposure-induced delays in fetal bone formation that contribute to fetal growth restriction and manifest as a decrease in crown–rump length, no significant ossification defects were observed in term fetuses as a result of exposure to any of the treatment paradigms utilized in the present study (data not shown) – regardless of the exposure period (pre-/peri-implantation gds 1–5, post-implantation gds 6–18 or throughout gestation gds 1–17) or the type of cigarette smoke (mainstream/sidestream vs. sidestream only) to which the dams were exposed.

Cigarette smoke exposure during embryo/fetal development has been linked to infant/childhood behavioral impairments and cognitive deficits (National Cancer Institute, 1999; Yolton et al., 2005). In the current study, fetal brains were harvested and weighed for all experimental smoke exposure paradigms. Exposures to both mainstream/sidestream cigarette smoke throughout gestation (gds 1–17) and to sidestream smoke during the pre-/peri-implantation period (gds 1–5) resulted in a statistically significant decrease in fetal brain weight. As there were no significant changes in brain weight:body weight ratio between the sham- and smoke-exposed groups, the observed decreases in brain weight may reflect a more generalized decrease in fetal organ size that is coincident with a reduction in body weight/length. This interpretation is consistent with the results of human studies in which it was found that infants of smoking women, besides having significant reductions in weight and crown–heel length, also have a significant overall decrease in fat-free mass (Lindsay et al., 1997). Reductions in fat-free mass would include muscle, visceral organs, bone and brain. Nevertheless, the observation that there was no statistically significant change in brain weight:body weight ratio between the sham- and smoke-exposed groups in the current study does not exclude the possibility that cigarette smoke exposure during gestation may have long-lasting deleterious effects on fetal brain development. Published observations have shown that, even in the absence of observable effects on gross morphological measures such as fetal weight, more subtle alterations on fetal brain development such as abnormal establishment of hippocampal layer cell size, thickness, and packing density occur as a result of prenatal nicotine exposure in rats (Navarro et al., 1989; Roy et al., 2002). In fact, recent studies from our laboratory, in which gene expression profiling with high-density microarrays was performed on either whole brain or hippocampal tissue of fetuses exposed to sidestream smoke on gds 6–18.5, demonstrated alterations in the expression of numerous genes involved in neuro-developmental, neuro-behavioral and cognitive processes (Singh et al., submitted for

publication; Horn et al., in preparation). In view of such intriguing observations, studies focused on addressing potential cellular and molecular alterations in the developing brain and attendant post-natal behavioral and cognitive deficits, following *in utero* exposures to cigarette smoke, are warranted.

In summary, data from this study demonstrated an association between abnormal fetal growth and both “active” or “passive” cigarette smoke exposure during gestation. This study also identified a temporal window of vulnerability during the pre-/peri-implantation period of embryonic development for *in utero* smoke exposure-induced low birth weight. Further, these studies document the establishment of a viable animal model with which to test hypotheses regarding the cellular and molecular mechanisms underlying gestational tobacco smoke exposure-induced low birth weight.

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Fig. 1.

Developmental periods of *in utero* cigarette smoke exposure. Pregnant C57BL/6J mice were exposed to either ambient air (sham) or cigarette smoke for 6 h a day throughout three periods of gestation, with assessment of reproductive and fetal outcomes on gestational day 18.5: pre-/peri-implantation (gestational days 1–5), post-implantation (gestational days 6–18) and throughout gestation (gestational days 1–17). Implantation (note asterisk) occurs between days 4 and 5 of gestation in the mouse.

Fig. 2.

Gestational cigarette smoke exposure induces intrauterine growth retardation. Pregnant C57BL/6J females were exposed to either ambient air (sham) or mixed mainstream/sidestream cigarette smoke on days 1–17 of gestation. Reproductive and fetal outcomes were subsequently assessed on day 18.5 of gestation. Since the dam was the smoke-exposed entity, litters were regarded as the experimental unit. Hence, individual fetal weight (Panel A) or crown–rump length (Panel B) was averaged and plotted in the scattergrams on a per litter basis. Results revealed a statistically significant decrease in body weight (Panel A) and crown–rump length (Panel B) in the cigarette smoke-exposed fetuses compared to the sham-exposed controls. Panels (C) and (D) show a representative sham-exposed fetus (Panel C) (weight 1.15 g; crown–rump length 28 mm) and a smoke-exposed fetus (Panel D) (weight: 1.08 g; crown–rump length 26 mm) on gestational day 18.5. The representative fetuses reflect the 6% reduction in weight observed following cigarette smoke exposure on gestational days 1–17. Reproductive and fetal outcomes for this exposure cohort, including fetal weight and crown–rump length are presented in Table 3.

Fig. 3. “Active” or “Passive” cigarette smoke exposure during the pre-/peri-implantation period of developmental affects fetal growth. C57BL/6J dams were exposed to either ambient air (sham), mixed mainstream/sidestream (“active”) smoke, or sidestream only (“passive”) smoke from days 1 to 5 of gestation. Reproductive and fetal outcomes were subsequently assessed on day 18.5 of gestation. Since the dam was the smoke-exposed entity, litters were regarded as the experimental unit. Hence, individual fetal weight (Panels A and C) or crown-rump length (Panels B and D) was averaged and plotted in the scattergrams on a per litter basis. Results revealed a statistically significant decrease in body weight (Panels A and C) and crown-rump length (Panels B and D) in both the “active” (upper panels) and “passive” (lower panels) smoke-exposed fetuses when compared to the sham-exposed controls. Reproductive and fetal outcomes for the “active” and “passive” smoke exposure cohorts, including fetal weight and crown-rump length, are presented in Tables 4 and 6, respectively.

Table 1

Chamber conditions for cigarette smoke- and sham-exposed dams^a, mainstream/sidestream cigarette smoke exposure, exposure periods: gestational days 6–18, 1–17, 1–5

Condition	Sham chamber ^b	CSE ^c chamber ^b
Carbon monoxide (ppm)	ND ^d	128.0 ± 3.0
Humidity (% RH)	31.8 ± 4.9	38.9 ± 5.9
Temperature (°C)	22.9 ± 0.1	21.9 ± 0.4
Total suspended particulates (mg/m ³)	0.2 ± 0.1	29.2 ± 2.6

^a Chamber measurements were monitored on a daily basis: immediately prior to, and then twice during, the actual exposure period.

^b Data for chamber conditions are reported as mean ± S.E.M.

^c CSE, cigarette smoke exposure.

^d ND, not detected.

Table 2

Reproductive and fetal outcomes^a, mainstream/sidestream cigarette smoke exposure, gestational days 6–18 exposure period

Outcome measure	Sham ^b	CSE ^{b,c}
Number of implantations	8.9 ± 0.3	7.9 ± 0.7
Number of resorptions	0.4 ± 0.2	0.8 ± 0.2
Number of viable fetuses	8.5 ± 0.3	7.1 ± 0.8
Number of abnormalities	0.2 ± 0.2	0.3 ± 0.2
Fetal weight (g)	1.15 ± 0.02	1.15 ± 0.03
Crown–rump length (mm)	28.5 ± 0.3	27.4 ± 0.3 ^d
Brain weight (mg)	81.1 ± 1.7	76.5 ± 3.7

^a Reproductive and fetal outcomes were assessed on day 18.5 of gestation.

^b Outcomes data are reported as mean ± S.E.M. for each measure (*n* = 10–11 litters per treatment group).

^c CSE, cigarette smoke exposure.

^d Smoke exposure on gestational days 6–18 resulted in a statistically significant decrease in fetal crown–rump length (*p* < 0.05).

Table 3

Reproductive and fetal outcomes^a, mainstream/sidestream cigarette smoke exposure, gestational days 1–17 exposure period

Outcome measure	Sham ^b	CSE ^{b,c}
Number of implantations	7.7 ± 0.4	8.5 ± 0.2
Number of resorptions	0.4 ± 0.2	0.9 ± 0.3
Number of viable fetuses	7.4 ± 0.5	7.6 ± 0.4
Number of abnormalities	0.0 ± 0.0	0.1 ± 0.1
Fetal weight (g)	1.15 ± 0.03	1.08 ± 0.02 ^d
Crown–rump length (mm)	28.4 ± 0.3	27.1 ± 0.2 ^d
Brain weight (mg)	80.6 ± 1.1	75.6 ± 1.3 ^d

^a Reproductive and fetal outcomes were assessed on day 18.5 of gestation.

^b Outcomes data are reported as mean ± S.E.M. for each measure (*n* = 14–17 litters per treatment group).

^c CSE, cigarette smoke exposure.

^d Smoke exposure on gestational days 1–17 resulted in statistically significant decreases in fetal weight, crown–rump length, and brain weight (*p* < 0.05).

Table 4

Reproductive and fetal outcomes^a, mainstream/sidestream cigarette smoke exposure, gestational days 1–5 exposure period

Outcome measure	Sham ^b	CSE ^{b,c}
Number of implantations	8.2 ± 0.4	8.9 ± 0.2
Number of resorptions	1.2 ± 0.3	0.7 ± 0.2
Number of viable fetuses	7.0 ± 0.4	8.2 ± 0.3 ^d
Number of abnormalities	0.3 ± 0.1	0.4 ± 0.2
Fetal weight (g)	1.18 ± 0.02	1.10 ± 0.01 ^d
Crown–rump length (mm)	28.7 ± 0.2	27.4 ± 0.2 ^d
Brain weight (mg)	81.2 ± 1.7	77.2 ± 0.9

^a Reproductive and fetal outcomes were assessed on day 18.5 of gestation.

^b Outcomes data are reported as mean ± S.E.M. for each measure (*n* = 12–13 litters per treatment group).

^c CSE, cigarette smoke exposure.

^d Smoke exposure on gestational days 1–5 resulted in statistically significant decreases in crown–rump length and fetal weight, and an increase in the number of viable fetuses (*p* < 0.05).

Table 5

Chamber conditions for cigarette smoke- and sham-exposed dams^a, sidestream cigarette smoke exposure, gestational days 1–5 exposure period

Condition	Sham chamber ^b	CSE ^c chamber ^b
Carbon monoxide (ppm)	ND ^d	35.7 ± 0.8
Humidity (% RH)	27.2 ± 2.0	28.2 ± 2.5
Temperature (°C)	22.5 ± 0.2	22.1 ± 0.2
Total suspended particulates (mg/m ³)	0.04 ± 0.04	5.99 ± 0.48

^a Chamber measurements were monitored on a daily basis: immediately prior to, and then twice during, the actual exposure period.

^b Data for chamber conditions are reported as mean ± S.E.M.

^c CSE, cigarette smoke exposure.

^d ND, not detected.

Table 6Reproductive and fetal outcomes^a, sidestream cigarette smoke exposure, gestational days 1–5 exposure period

Outcome measure	Sham ^b	CSE ^{b,c}
Number of implantations	9.1 ± 0.3	9.3 ± 0.2
Number of resorptions	0.5 ± 0.2	0.9 ± 0.3
Number of viable fetuses	8.6 ± 0.3	8.3 ± 0.3
Number of abnormalities	0.1 ± 0.1	0.1 ± 0.1
Fetal weight (g)	1.17 ± 0.01	1.13 ± 0.01 ^d
Crown–rump length (mm)	28.4 ± 0.3	27.6 ± 0.2 ^d
Brain weight (mg)	9.0 ± 0.7	75.5 ± 1.1 ^d

^a Reproductive and fetal outcomes were assessed on day 18.5 of gestation.^b Outcomes data are reported as mean ± S.E.M. for each measure (*n* = 15 litters per treatment group).^c CSE, cigarette smoke exposure.^d Smoke exposure on gestational days 1–5 resulted in statistically significant decreases in fetal body weight, fetal crown–rump length, and fetal brain weight following sidestream smoke exposure on gestational days 1–5 (*p* < 0.05).