

Evidence for Carbon Monoxide as the Major Factor Contributing to Lower Fetal Weights in Rats Exposed to Cigarette Smoke

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Received September 18, 2007; accepted December 11, 2007

One of the major effects of cigarette smoking during pregnancy is bearing a child with lower birth weight. It has previously been demonstrated under experimental conditions in rats that exposure to reference cigarette smoke results in reduced birth weight (E. L. Carmines *et al.*, 2003, *Toxicol. Sci.* 75, 134–147; C. L. Gaworski *et al.*, 2004, *Toxicol. Sci.* 79, 157–169). The role of various smoke constituents on lower birth weight was evaluated by exposing time-pregnant Sprague-Dawley rats at the concentrations found in cigarette smoke. The rats were exposed for 2 h/day 7 days/week by nose-only inhalation. The target concentrations were designed to produce the same plasma levels of biomarkers as exposure to 2R4F reference cigarette smoke at a concentration of 600 mg/m³ total particulate matter. The smoke constituents evaluated included carbon monoxide (CO), nicotine, and a mixture of aldehydes (acrolein, acetaldehyde, and formaldehyde). The smoke constituents were tested individually as well as in mixtures to evaluate potential interactions. Exposure to cigarette smoke during gestation produced a reduction in both maternal body weight gain and fetal weights. Exposure to nicotine reduced maternal body weight gain but had no effect on fetal weight. Exposure to CO had no effect on maternal body weight gain but reduced fetal weight to a degree comparable to cigarette smoke. Exposure to a mixture of aldehydes (acrolein, acetaldehyde, and formaldehyde) had no effect on either maternal body weight gain or fetal weight. Exposure to mixtures of nicotine and CO or nicotine, CO, and aldehydes did not demonstrate any interactions. The results of this study suggest that the observed reduction in fetal weight after exposure to cigarette smoke in rats is due to CO toxicity and not nicotine toxicity.

Key Words: cigarette smoke; low birth weight; nicotine; carbon monoxide.

In the 1964 Surgeon General's report, it was stated that "Women who smoke cigarettes during pregnancy tend to have babies of lower birth weight" (US Public Health Service, 1964). Low birth weight has been extensively investigated since 1964, and it is now clear that there is a direct relationship between the number of cigarettes smoked, the carbon

monoxide (CO) concentration, or the tar yield of the cigarettes and the relative risk for low birth weight. However, despite numerous investigations, the mechanism for the effect remains elusive.

Previous studies in laboratory animals have produced mixed results concerning an association between cigarette smoke and fetal effects. In an early study, Essenberg *et al.* (1940) produced reduced birth weights in rats using a crude whole-body smoke exposure apparatus. Reznik and Marquard (1980) produced a reduction in birth weight by whole-body exposure of rats to the smoke of 30 cigarettes over 7–11 min for up to four smoking cycles per day. Reckzeh *et al.* (1975) used nose-only exposure to study the effects of diluted mainstream smoke from 30 cigarettes for 9–10 min twice a day. Maternal body weight gain was reduced by smoke exposure compared to controls, but there was no effect on litter weights. Bertolini *et al.* (1982) studied the reproductive effects of commercial cigarettes in rats by using whole-body exposure to smoke for 15 min/day during gestation. Dam weight gain was significantly reduced, but there was no effect on pup weight. Using repetitive 8-min exposures in mice and variable exposure duration during discrete periods of gestation, Wagner *et al.* (1972) found that while maternal body weight gain was significantly reduced during gestation, there was no effect on fetal weights. In another study in mice, after nose-only exposure to the smoke of six commercial cigarettes for 10 min per day, a reduction in fetal body weight and delayed development as indicated by a reduction in the number of skeletal ossification centers was observed (Seller and Bnait, 1995). Mice were also used in another whole-body exposure using the smoke of one and one-half University of Kentucky reference cigarettes (specific type unknown) from gestation days 6 to 17 (Peterson *et al.*, 1981). There was no effect on fetal mortality, weight, or crown-rump length. In a recent study, Farkas *et al.* (2006) attempted to expose rats to cigarette smoke at levels designed to deliver plasma nicotine levels close to those observed in smokers. Unfiltered high-yield reference cigarettes (2R1) were smoked in a nonstandard manner (40 ml puff lasting 2.4 s every minute). Plasma nicotine and carboxyhemoglobin concentrations were measured, but no analysis of the smoke was reported. The unit of smoke

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TABLE 1
Study Design Used to Examine the Effect of Cigarette Smoke or Selected Smoke Constituents on Fetal Weight

Group	Target concentration (mg/m ³)	No. of animals	
		Core study	Sentinels
Sham control	0	27	5
CO	687	27	5
Nicotine	20	27	5
Aldehydes	25	27	5
Formaldehyde	1		
Acrolein	4		
Acetaldehyde	40		
CO + nicotine	687 + 20	27	5
CO + nicotine + aldehydes	687 + 20 + 25	27	5
2R4F cigarette smoke	600 ^a	27	5
Cage control	—	27	5

^amg TPM/m³.

exposure was the inhaled volume of smoke per day (ml/day). The authors attempted to match the plasma nicotine levels observed in humans but did not match the carboxyhemoglobin levels. Exposure to high levels of smoke induced maternal toxicity and fetal mortality. Lower levels of smoke did not affect maternal weight gain but produced a lower birth weight. Hussein *et al.* (2007) followed up on the work of Farkas exposing rats to nicotine via osmotic mini pumps. They found that high levels of nicotine had no effect on birth weight. The interpretation of these reported studies is difficult because the types of cigarettes tested are generally not described, the smoke yield of the cigarettes is not reported, and the exposure conditions are not well documented. Recently, our laboratory performed a thorough evaluation of the potential effects of exposure to well-characterized reference cigarette smoke (Carmines *et al.*, 2003; Gaworski *et al.*, 2004) and found that the main effect was a reduction in maternal, fetal, and birth weights. The goal of this research was to evaluate selected smoke constituents that might be contributing to the reduced fetal weight. Exposure to the smoke constituents was designed to mimic exposure during smoking.

MATERIALS AND METHODS

Study design. Table 1 summarizes the study design. The goal of the study was to evaluate the role of selected smoke constituents on fetal weight. Groups of 32 time-pregnant rats were exposed by nose-only inhalation to test atmospheres of nicotine, CO, a mixture of aldehydes (acrolein, acetaldehyde, and formaldehyde), or combinations of smoke constituents or diluted mainstream cigarette smoke for 2 h (1 h test atmosphere/30 min air/1 h test atmosphere) per day from gestation days 6–19. There were sham (exposed to filtered air only) and cage control groups. The cage control group did not have access to food and water during the exposure time. Cesarean sections were performed, and the fetuses were evaluated on gestation day 20. Separate groups of sentinel animals undergoing exposure (gestation days 6–20) were included in

each group and used for biomarker determination. Exposure concentrations and time periods were based on previous experience with nose-only cigarette smoke exposures (Carmines *et al.*, 2003).

Test atmosphere generation. Smoke was generated on an automatic 30-port carousel smoking machine designed for continuous smoke generation over several hours (Reininghaus and Hackenberg, 1977, modified, type: SM85). The machine operates in basic conformity with the International Organization for Standardization (ISO) 3308 (International Organization for Standardization, 1991a) standard smoking protocol. The key parameters were a puff volume of 35 ml, a puff duration of 2 s, and a puff frequency of 1 puff per cigarette every 60 s. The carousel rotated in steps at a speed of one revolution per minute (each of the 30 cigarettes puffed once for 2 s) resulting in a constant stream of mainstream smoke. Some minor deviations from the ISO standard, such as rectangular puff profile, free (open-end) smoking, and air velocity, were necessary for technical reasons and were not considered to substantially affect the smoke composition. At the puffing port, cigarettes were lit with a halogen spot lamp; butts were ejected at a mean butt length of 35 mm. Near the smoking position, the continuously generated smoke was diluted with filtered conditioned fresh air. The 2R4F reference cigarettes were obtained from the Tobacco and Health Research Institute at the University of Kentucky (Diana and Vaught, 1990). All cigarettes were conditioned according to ISO standard 3402 (International Organization for Standardization, 1991b) before being smoked.

The CO test atmosphere was generated by dilution of CO from a compressed gas cylinder (Mittler Supply, South Bend, IN). The nicotine atmosphere was generated by bubbling air through liquid nicotine (Sigma Aldrich Company, St Louis, MO) and then dilution with air. The aldehyde mixture was generated by individually adding each aldehyde into air mixing chamber of the inhalation chamber. The acrolein (Sigma Aldrich Company) and acetaldehyde (Sigma Aldrich Company) atmospheres were generated by addition to gasbags at a specified concentrations and then dilution with air. Formaldehyde was generated by heating paraformaldehyde (Sigma Aldrich Company) and then dilution with air.

Animals and animal care. Care and use of the animals were in conformity with the American Association for Laboratory Animal Science Policy on the Humane Care and Use of Laboratory Animals (AAALAC, 1991). Pregnant Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY). After randomization by body weight, each animal was single housed in plastic shoebox cages (Lab Products, Maywood, NJ) with absorbent hardwood chip bedding during nonexposure periods. The animal rooms were maintained at 20°C–24°C and 40–70% relative humidity. Rats were provided with Certified Rodent Meal #5002 (PMI Nutrition International, Inc., Brentwood, MO) and City of Chicago municipal tap water *ad libitum* except during inhalation exposures.

Exposure. The rats were nose-only exposed in Canon-style exposure chambers (Lab Products) using polycarbonate restraint tubes matching their body size (CH Technologies, Westwood, NJ). The daily routine was a 1-h exposure, followed by one-half hour air period, and followed by another 1-h exposure.

Analytical characterization of the test atmospheres. Smoke total particulate matter (TPM) concentrations were determined at least twice a day (once per hour) using a gravimetric filter collection method. The sampling train consisted of a preweighed filter connected to a constant flow vacuum pump. Smoke particles were collected on preweighed 47-mm fiberglass filter disks (Pall Corporation, Ann Arbor, MI) from inhalation exposure ports at flow rates matching the smoke test atmosphere delivery rate. The aerosol mass collected on the filter was weighed and a dry gas meter was used to measure the corresponding sample volume. The weight-to-volume ratio was determined to provide the TPM concentration in the test atmosphere. The nicotine concentration was determined twice per group per exposure week by trapping the exposure atmosphere in a sulfuric acid-impregnated silica gel tube (Extrelut NT; Merck, Darmstadt, Germany) and eluted from the tube with an *n*-butyl

TABLE 2
Concentrations (mg/m³) of Smoke Constituents in the Test Atmospheres^a

Group	Target concentration	CO	Nicotine	Formaldehyde	Acetaldehyde	Acrolein	TPM
Sham control	0	2 ± 2.2	NA ^b	NA	NA	NA	NA
CO	687	735 ± 11.6	NA	NA	NA	NA	NA
Nicotine	20 mg/m ³	NA	18.18 ± 1.88	NA	NA	NA	NA
Aldehyde	Aldehyde mixture ^c	NA	NA	1.00 ± 0.41	40.83 ± 9.32	4.02 ± 1.03	NA
CO + nicotine	CO (687 mg/m ³), nicotine (20)	727 ± 9.2	19.38 ± 1.94	NA	NA	NA	NA
CO + nicotine + aldehydes	CO (687 mg/m ³), nicotine (20), aldehyde mixture ^c	736 ± 5.0	17.82 ± 1.52	0.99 ± 0.28	39.20 ± 8.14	4.50 ± 0.83	NA
2R4F cigarette smoke	600 ^d	713 ± 35.0	33.85 ± 2.46 ^e	0.89 ± 0.10 ^e	36.64 ± 2.34 ^e	3.46 ± 0.15 ^e	623 ± 34.4
Cage control		NA	NA	NA	NA	NA	NA

^aMean ± SD; N = 18.

^bNA = not applicable.

^cAldehyde mixture of formaldehyde (1 mg/m³), acrolein (4 mg/m³), and acetaldehyde (40 mg/m³).

^dmg TPM/m³.

^eN = 6.

acetate solution. The amount of nicotine collected was determined by gas chromatography (6890 series; Agilent Technologies, Wilmington, DE) equipped with a nitrogen-phosphorus detector and an HP-5 column (30 m long × 0.32 mm diameter). CO concentrations were monitored in each exposure chamber continuously with a dedicated infrared gas analyzer (Model ZRH-1; California Analytical Instruments, Orange, CA) by drawing filtered atmosphere through the gas analyzer. The gas analyzers were calibrated with gas standards, and the calibration was checked prior to the initiation of each daily exposure. Aldehydes were trapped in acetonitrile as 2,4-dinitrophenylhydrazine derivatives. The sample solution was stabilized by addition of pyridine and analyzed by high-performance liquid chromatography (Agilent Technologies, model 1100 HPLC equipped with a model 1313 auto sampler, a model 1354 quaternary pump with degasser, and a model 1314A variable wavelength ultraviolet detector set to 365 nm) using water-methanol mobile (45:55%) phase.

Biomonitoring. Biomonitoring was conducted on the sentinel animals and their pups to determine exposure. Within 5 minutes after being removed from the exposure chamber (to prevent dissociation of COHb), sentinels were anesthetized with 70% CO₂/30% air and bled from the retro-orbital sinus for determination of COHb and nicotine. Blood from fetal sentinels was collected following decapitation and pooled on a litter basis for nicotine and cotinine determination on gestation day 20. COHb was analyzed immediately after collection using an IL-482 CO-oximeter (Instrumentation Laboratory, Lexington, MA), while the serum for nicotine and cotinine determination was stored at -70°C until all samples could be analyzed by radioimmunoassay methods as described by Van Vunakis *et al.* (1987).

In-life and postmortem evaluation. The rats were observed twice a day during the exposure period for morbidity and mortality. Animals received clinical observations concurrent with body weight and food consumption measurements on gestation days 4, 6, 9, 12, 15, 18, and 20 and prior to scheduled termination. The day a sperm-positive smear was collected was considered gestation day 0. Dams were euthanized by CO₂ asphyxiation on gestation day 20, and cesarean sections were conducted. The uterus was removed from the dam, trimmed free of excess adherent tissue, and weighed, with the ovaries, prior to removal of the fetuses. After weighing, the corpora lutea were counted and recorded for the left and right ovaries. Each uterine horn was inspected for resorptions and fetal deaths. Resorptions were counted and classified as an early resorption (placenta only), late resorption (placenta and attached fetal parts), early death (fetus weighing less than 0.8 g) or late death (fetus weighing more than 0.8 g).

Fetal evaluation. Each live fetus was counted and received a gross external morphologic examination. Live fetuses from each litter were sexed and

weighed. Since previous studies had indicated no teratological effects of cigarette smoke (Carmines *et al.*, 2003; Gaworski *et al.*, 2004), no further evaluation was performed.

Statistical procedures. Means and SDs were calculated for all measured parameters. Since the cage control group was not subjected to potential stress factors from the nose-only exposure, statistical analyses included only the sham and smoke- or test atmosphere-exposed groups. Body weights, body weight gains, food consumption, and organ weight data were analyzed by ANOVA followed, where appropriate, by Dunnett test using SYSTAT (SPSS, Inc., Chicago, IL). The litter was the sampling unit (Weil, 1970). Comparison of litter (fetal) body weight data was analyzed by an ANOVA; the litter was the unit of observation. For fetal data, a one-factor (i.e., treatment group) ANOVA was used for mean total males and females per litter, mean corpora lutea, mean total, live and non-live (resorptions and deaths) implants, mean percent live and non-live implants, and mean percent preimplantation loss. A significance level of $p \leq 0.05$ was used for all comparisons.

RESULTS

Exposure

Table 2 lists the respective atmosphere concentrations for each exposure group. The measured daily mean smoke TPM exposure concentration for the entire exposure period was 623 mg TPM/m³, which was within 5% of the target 600 mg TPM/m³ target level. The corresponding CO and nicotine levels for the smoke groups were 723 and 33.85 mg/m³, respectively. The formaldehyde, acetaldehyde, and acrolein levels in the smoke-exposed group were 0.89, 36.64, and 3.46 mg/m³, respectively. Preliminary studies with the nicotine vapor generation system indicated that nicotine blood levels from nicotine vapor exposure groups were substantially higher than the corresponding levels from smoke exposure groups. Consequently, the nicotine concentration in the atmosphere for the nicotine exposure groups was reduced to match the actual blood level obtained from smoke exposure groups (see the biomarker data below). The nicotine concentration in the

nicotine-containing exposure groups ranged from 17.8 to 19.4 mg/m³ compared to 33.9 mg/m³ for smoke. The CO exposure levels ranged from 727 to 736 mg/m³ compared to 713 mg/m³ for smoke. The respective formaldehyde, acetaldehyde, and acrolein levels ranged from 0.99 to 1.00, 39.20 to 40.83, and 4.02 to 4.50 mg/m³. The levels of the individual chemical constituents were representative of the levels occurring in the test smoke.

Biomonitoring

Exposure to smoke was demonstrated by plasma levels of nicotine in the dams and pups (Fig. 1). The nicotine and nicotine + CO groups had essentially the same plasma nicotine levels as the smoke-exposed group. The nicotine + CO + aldehydes group had about 40% lower plasma nicotine levels than the nicotine exposure group. The cause of this is unknown. COHb was measured in the sentinel dams at gestation day 20 (Fig. 2). The groups exposed to CO had COHb levels similar to those in the smoke-exposed group. Because of the time delay necessary for cesarean sectioning, the amount of blood available, and the elimination half-life of COHb (42 min for adult rats; Ayres *et al.*, 1989), it was not possible to measure fetal COHb levels.

Maternal Effects

No animals died during the study. The clinical observations in the females were similar in all sham and treated groups and included diarrhea, salivation, wet inguinal fur, and red material around the eyes and nose. These observations were not seen in the cage controls and were attributed to the restraints of nose-only exposure system. Maternal body weights are illustrated in Figure 3. The smoke-exposed animals appeared to gain less weight than the other groups reaching statistical significance at

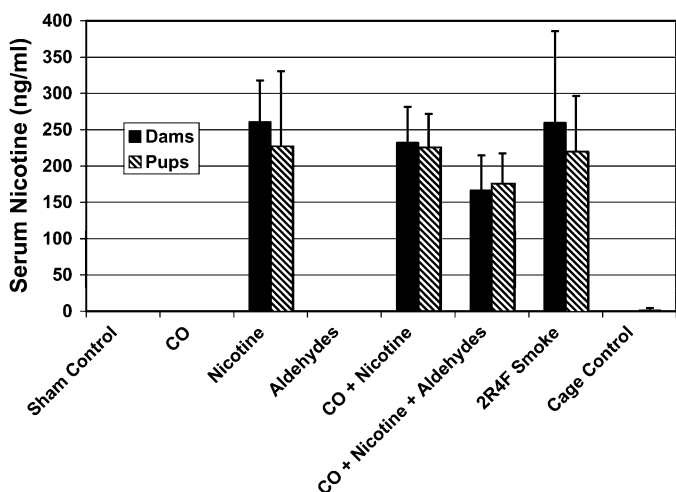


FIG. 1. Serum nicotine concentrations in dams and pups on gestation day 20 after exposure to cigarette smoke or selected smoke constituents (mean \pm SD, $N = 8$ for dams and 6–8 for pups).

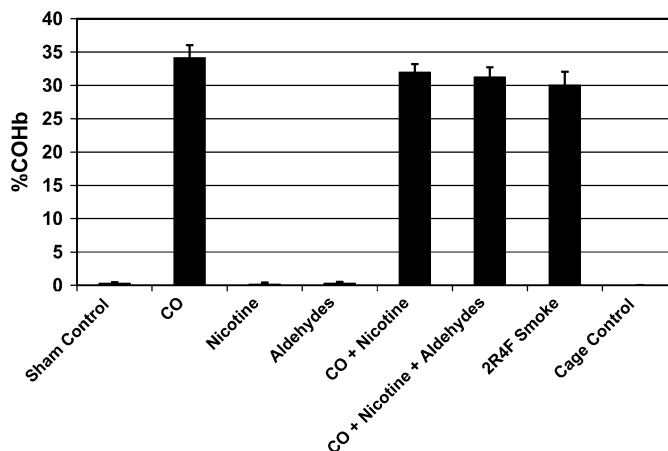


FIG. 2. Carboxyhemoglobin in dams on gestation day 20 after exposure to cigarette smoke or selected smoke constituents (mean \pm SD; $N = 8$).

gestation day 18. Food consumption was reduced only in the smoke-exposed animals. Maternal body weight gains are shown in Figure 4. All the groups exposed to nicotine (including the 2R4F smoke-exposed animals) had statistically significant lower body weight gains. The body weight gain of the aldehyde mixture group and the CO-only group was not statistically different from the sham. Uterine weights were significantly reduced in the CO alone and 2R4F smoke-exposed groups, only (Table 3). Corrected terminal body weight gains (maternal body weight change during gestation minus the uterine weight) were significantly reduced in nicotine-containing groups. The CO exposure group had a significantly increased terminal body weight gain compared to the sham-exposed group. Food consumption was not affected. Thus, all the nicotine-containing atmospheres (including smoke) showed reduced maternal body weight gain during gestation but CO had no detrimental effect on body

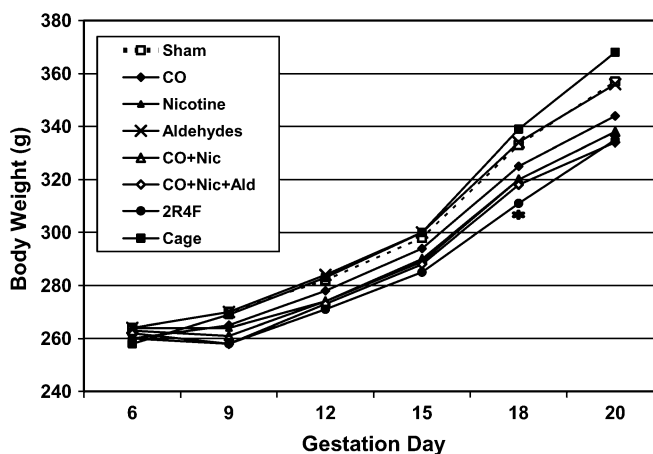


FIG. 3. Maternal body weights corrected for nonpregnant animals after exposure to smoke or selected smoke constituents. Error bars have been omitted for clarity (mean, $N = 23$ –27) * $p \leq 0.05$.

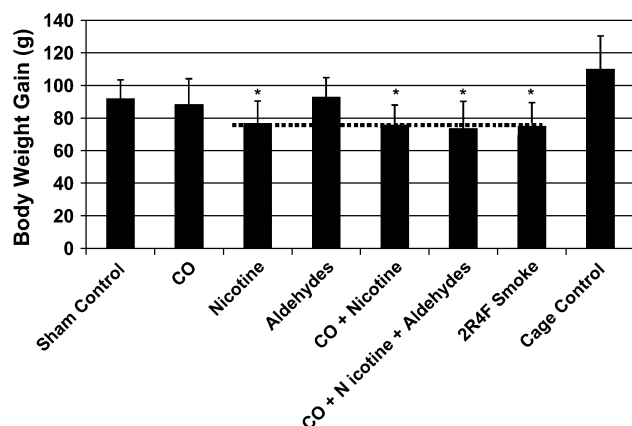


FIG. 4. Body weight gain at gestation day 20 after exposure to cigarette smoke or selected smoke constituents; mean \pm SD; * $p \leq 0.05$, $N = 23-26$.

weight gain. Gestational data, including corpora lutea, resorptions, implantation sites, live and dead fetuses, and mortality are presented in Table 4. Smoke inhalation and exposure to the smoke constituents did not affect these parameters.

Fetal Effects

Maternal exposure to cigarette smoke or the CO-containing constituent test groups reduced the fetal weight by about 0.5 g (~13%), Figure 5. Exposure to nicotine or the aldehyde mixture had no effect on fetal weight. There were no effects on the sex ratio (Table 4) or the number of external malformations (Table 5). The observed malformations are known to occur in this strain of rat. No external variations were observed.

DISCUSSION

Epidemiological investigations have established a relatively consistent association between maternal smoking and the risk

of lower birth weight and small for gestational age births in humans (US DHHS, 2001). The Surgeon General recommends pregnant women not to smoke. Cigarette labels warn pregnant women of the risk of low birth weight. Unfortunately, 12–22% of pregnant women continue to smoke during pregnancy (US DHHS, 2001). Previous animal studies with cigarette smoke have been inconclusive as to the cause of reduced birth weight. We have demonstrated that exposure of rats to well-controlled and -characterized cigarette smoke before and during gestation resulted in lower fetal and birth weight (Carmines *et al.*, 2003; Gaworski *et al.*, 2004). There is much speculation on the cause of the lower birth weight. There are three general hypotheses for the reduced birth weight observed in women who smoke during pregnancy. The first is nicotine toxicity or a pharmacologic effect of nicotine such as vasoconstriction of the placenta resulting in reduced blood flow to the fetus (Lambers and Clark, 1996); disruption in lipid metabolism resulting in diminished nutrient supply (Mosier *et al.*, 1974); or reduction in food consumption by the mother resulting in diminished nutrient supply. The second hypothesis is that formation of COHb inhibits the amount of oxygen available to the fetus (Lambers and Clark, 1996). The third hypothesis is that one or a combination of the 4000 other chemicals in smoke is causing the toxicity.

Three different approaches were used to evaluate the potential causes of the lower birth weight. Each was focused on trying to generate conditions that were identical to those that occurred during controlled exposure to cigarette smoke. The first was to expose pregnant rats to nicotine at a concentration equivalent to that which occurs in exposure to smoke. Nicotine vapor was used as the test material. Initial studies indicated that the serum levels of nicotine after exposure to nicotine vapor were substantially higher than that after exposure to cigarette smoke containing nicotine in the particulate phase. This is not unexpected since absorption of the gaseous (vapor) nicotine should be more direct when compared to absorption of the

TABLE 3
Body Weight and Uterine Weight Data after Exposure to Cigarettes Smoke or Selected Smoke Constituents

	Sham control	CO	Nicotine	Aldehydes	CO + nicotine	CO + nicotine + aldehydes	2R4F	Cage control
Mean maternal body weight at termination (g) ^a	357 \pm 31.3	348 \pm 31.1	343 \pm 24.8	356 \pm 21.5	338 \pm 29.7	337 \pm 31.7	335 \pm 27.3*	368 \pm 36.8
Maternal body weight change during gestation (g) ^a	92 \pm 11.8	88 \pm 16.1	77 \pm 13.9*	93 \pm 12.3	76 \pm 12.4*	74 \pm 16.8*	76 \pm 14.4*	110 \pm 20.5
Food consumption day 6–20 (g) ^a	261 \pm 30.8	257 \pm 24.6	251 \pm 25.6	267 \pm 28.4	254 \pm 38.5	240 \pm 30.1	236 \pm 22.8*	282 \pm 26.0
Mean uterine weight (g) ^a	76 \pm 13.7	66 \pm 12.5*	71 \pm 14.3	78 \pm 9.9	70 \pm 8.1	69 \pm 11.9	64 \pm 12.3*	76 \pm 18.3
Corrected terminal body weight gain (g) ^a	15 \pm 9.0	22 \pm 7.3*	6.1 \pm 9.9*	15 \pm 9.6	6 \pm 8.1*	4 \pm 11.0*	11 \pm 9.7	34 \pm 12.8

^aMean \pm SD.

*Significantly different from sham control ($p \leq 0.05$).

TABLE 4
Gestational Data from Rats Exposed to Cigarette Smoke or Selected Smoke Constituents

	Sham control	CO	Nicotine	Aldehydes	CO + nicotine	CO + nicotine + aldehydes	2R4F	Cage control
No. on study	27	27	27	27	27	27	27	27
Gravid females	23	26	25	25	26	27	24	26
Viable litters	23	25	24	25	26	26	24	26
Corpora lutea per litter ^a	15 ± 2.6	15 ± 3.8	15 ± 2.4	16 ± 2.3	15 ± 2.5	16 ± 2.8	15 ± 1.7	16 ± 3.6
Implantation sites per litter ^a	13 ± 2.4	13 ± 3.2	13 ± 3.2	14 ± 1.9	14 ± 1.7	14 ± 2.0	14 ± 2.3	13 ± 3.0
Preimplantation loss per litter ^a	2 ± 1.9	2 ± 2.6	3 ± 2.9	2 ± 1.5	2 ± 2.3	2 ± 2.3	2 ± 2.7	3 ± 3.5
Live fetuses per litter ^a	13 ± 2.5	12 ± 3.4	11 ± 4.0	13 ± 2.0	13 ± 1.7	13 ± 3.3	13 ± 2.6	12 ± 3.5
Resorptions per litter ^a	0 ± 0.7	1 ± 0.9	1 ± 1.4	1 ± 0.6	0 ± 0.5	1 ± 1.8	1 ± 0.8	1 ± 1.0
Early	9	20	22	16	7	14	19	16
Late	0	1	5	0	0	9	1	3
Total	9	21	27	16	7	23	20	19
Dead fetuses	0	0	9	0	0	0	0	2
Mean fetal weight (g) ^a	3.99 ± 0.331	3.55 ± 0.377*	3.89 ± 0.581	3.90 ± 0.295	3.43 ± 0.267*	3.42 ± 0.342*	3.33 ± 0.314*	4.06 ± 0.309
Males ^a	4.08 ± 0.403	3.60 ± 0.399*	3.96 ± 0.622	3.96 ± 0.324	3.52 ± 0.275*	3.49 ± 0.318*	3.43 ± 0.334*	4.17 ± 0.320
Females ^a	3.89 ± 0.342	3.48 ± 0.365*	3.79 ± 0.538	3.83 ± 0.302	3.33 ± 0.270*	3.34 ± 0.387*	3.24 ± 0.318*	3.98 ± 0.281
Fetal sex ratio (male:female, %)	51:49	50:50	51:49	51:49	50:50	51:49	47:53	54:46

^aMean ± SD.

*Significantly different from sham control ($p \leq 0.05$).

nicotine in the particulate phase of cigarette smoke. To compensate for this, the nicotine vapor concentration was reduced to match serum concentrations resulting from exposure to cigarette smoke. The serum studies on the dams and pups indicated that nicotine levels used were essentially the same as those produced by exposure to cigarette smoke. The nicotine levels in the rats after exposure to cigarette smoke were comparable to that one could expect in humans. Human smoking is an intermittent behavior that occurs throughout the day. This results in peaks and troughs of nicotine plasma levels (Benowitz *et al.*, 1982; Hukkanen *et al.*, 2005). Our exposure scenario was 60 min of smoke exposure with a 30-min break followed by another 60 min of smoke exposure. This pattern invariably does not duplicate normal human smoking. Our

exposure system was designed to minimize the stress (as indicated by the difference in body weight gain of the sham controls compared to the untreated cage controls) of the nose-only exposure system. While the nicotine pharmacokinetics are not exactly the same for rats and humans, the data presented here suggest that the rats received a dose of nicotine that could be equivalent to that smokers get on a daily basis. In this study, exposure to nicotine vapor alone at levels comparable to smoking reduced dam weight gain but had no effect on rat fetal weight. These results are not generally consistent with the literature on pure nicotine exposure. The nicotine literature suggests that it is teratogenic when administered to pregnant mice as a pure material at high levels (25 mg/kg) (Nishimura and Nakai, 1958). Lower levels (using a smokeless tobacco extract equivalent to 12 mg/kg) produced no deaths or deformities (Paulson *et al.*, 1989). In rats, nicotine causes a decrease in embryo growth, delays implantation, retards parturition, reduces the number of litters and of total young born, and produces an increase in mortality of the young during the nursing period (Becker and King, 1966; Becker *et al.*, 1968; Essenberg *et al.*, 1940; Hammer and Mitchell, 1979; Haworth and Ford, 1972; Thienes, 1960). It has been long known that the pharmacology and toxicology of tobacco smoking and the pharmacology and toxicology of nicotine are not identical and are often not comparable (Silvette *et al.*, 1962). Under conditions similar to that which occur during smoking in rats, nicotine had no effect on fetal weight.

The second approach was to expose animals to the same concentration of CO as in cigarette smoke. This was done by diluting bottled CO with air and exposing the animals. The maternal COHb data indicate that this approach was successful.

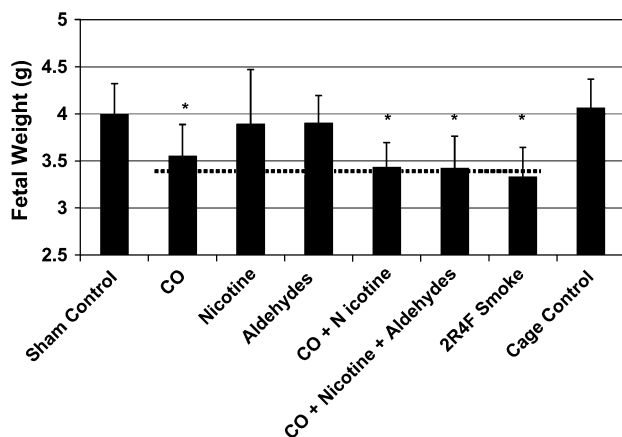


FIG. 5. Fetal weight after maternal exposure to cigarette smoke or selected constituents, mean ± SD; * $p \leq 0.05$.

The COHb level was about 30% immediately after exposure. The kinetics of COHb in rats and humans are different. Heavy cigarette smokers may have COHb levels greater than 12% (Scherer, 2006). It is generally accepted that cigarette smokers have COHb levels in the range of 4–6%. This is influenced by a number of variables including number of cigarettes smoked, different CO yields of cigarettes, smoking intensities, and different levels of physical activity. The human half-life of COHb while sleeping is estimated to be about 4–6 h, while when exercising vigorously it may be less than an hour (Wald and Howard, 1975). The half-life of COHb in rats is about 42 min at the 600-ppm exposure level (Ayres *et al.*, 1989). Thus, the rats in this study experienced a higher peak COHb, but humans have a much longer duration of effect. It should be noted that placental hemoglobin has a higher affinity for CO than maternal hemoglobin. Visnjevac and Mikov (1986) reported finding newborn COHb levels nearly one-third higher than those in their mothers. Leonard *et al.* (1989) reported a 40% increase in COHb levels. We were not able to measure the COHb levels in the fetuses in this study because of timing of the caesarian section and the need to sample blood immediately after exposure. We did measure it in a previous study and found the COHb level to be about 50% higher in the rat fetus (data not shown). Many authors (Bureau *et al.*, 1982; Leonard *et al.*, 1989; Visnjevac and Mikov, 1986; Wouters *et al.*, 1987) have correlated increased COHb and reduced birth weight in humans. The problem lies in the fact that smoking is a long-term exposure during gestation, and the reduced birth weight effect is a cumulative effect that develops over the full gestation term. Essentially, the clinical approach has been to take cord or fetal blood at birth and correlate it with reduced birth weight. The amount of COHb at birth is simply a measure of the CO exposure over the past 24 h and does not reliably predict the cumulative exposure over the full gestation term. While the levels of COHb are not the same in rats and humans, there does appear to be a strong correlation.

Our findings of reduced birth weights are consistent with the literature. Astrup *et al.* (1972) have previously demonstrated a direct relationship between CO intoxication and lower birth weights in rabbits. Exposure of laboratory animals to CO has resulted in developmental toxicity in the rat, rabbit, guinea pig, pig, and monkey (Schardein and Keller, 1989). In a unique smoke-related investigation, Reznik and Marquard (1980) exposed pregnant rats to either whole smoke or the gas phase of smoke. Whole smoke produced a greater effect on fetal birth weight than did comparable dosing with the gas phase, suggesting that CO alone was not the cause of low birth weight. Tachi and Aoyama (1983) exposed dams to cigarette smoke during the total gestation period and to 6-day segments. A separate group of dams was exposed to CO at the same concentration as was found in the smoke. Cigarette smoke reduced fetal weight with the largest effect being produced during the last 7 days of gestation. The CO-exposed group experienced a reduced fetal weight also but not to the extent of

the smoke-exposed group. The authors concluded that CO exposure was a contributor to the effects of cigarette smoke but not the sole factor responsible for the adverse effects. Our research indicates that under controlled exposure conditions, CO, at levels present in cigarette smoke, reduces fetal weight while having essentially no effect on dam weight. Interestingly, the combination of CO and nicotine almost perfectly matched the effects of cigarette smoke on dam weight and fetal weight, suggesting that the effects were additive and independent of each other.

The third approach was to expose animals to a mixture of aldehydes broadly similar to that occurs in cigarette smoke. Mixtures of the aldehydes and CO and nicotine were also tested to look at the combined effect. The testing hypothesis was that the aldehyde atmosphere could possibly be irritating and therefore alter respiration and/or produce maternal/fetal toxicity. No biomarker of aldehyde exposure was used. However, the concentrations of the aldehydes in the test atmospheres were very close to that observed for the smoke-exposed group. The aldehyde mixture had no effects on the dams or pups. Addition of aldehydes to CO and nicotine did not appear to affect the response to the individual chemicals.

While this study design was simple, the interpretation of the results was difficult because of the different effects of nicotine and CO. Nicotine exposure reduced the maternal body weight gain as measured by the corrected terminal body weight gain. This value is the result of subtracting out the weight of the uterus including the fetuses providing a corrected body weight gain during gestation. This corrected body weight gain provided an insight into the health of the dams. All the nicotine groups had statistically significant reduced terminal body weight gains suggesting maternal toxicity. CO exposure increased the corrected terminal body weight gain, suggesting that it had no long-lasting adverse effect on the dam. The combination of CO and nicotine produced a reduced terminal body weight gain consistent with nicotine exposure alone. This was also true for the nicotine + CO + aldehyde group. As indicated by the CO exposure alone, CO appeared to have no effect on body weight gain when combined with nicotine. In the smoke-exposed animals, there was a nonstatistically significant reduction in terminal body weight gain. It is not clear why the effect was not more pronounced. A more pronounced statistically significant reduction in corrected terminal body weight gain was previously observed after exposure to cigarette under similar conditions (Carmines *et al.*, 2003). Uterine weights were statistically significantly reduced in the CO-only and smoke groups. Fetal weights were significantly reduced in all the CO groups and in the smoke group. One might have expected that the uterine weights would be affected to the same degree as the fetal weights. It is not clear why they were not. One explanation might be that the measurement of uterine weights is somewhat inexact being influenced by the degree of trimming of the tissue at cesarean section and the amount of blood and the amount of amniotic

TABLE 5
Summary of External Malformations after Exposure to Cigarette Smoke or Selected Smoke Constituents

Group	Sham	CO	Nicotine	Aldehydes	CO + nicotine	CO + nicotine + aldehydes	2R4F	Cage
Litters examined	23	25	24	25	26	26	24	26
Fetuses examined	291	304	281	333	347	345	306	313
Micrognathia/agnathia								
Fetal incidence	0	0	0	0	0	1	0	0
Litter incidence	0	0	0	0	0	1	0	0
Umbilical hernia								
Fetal incidence	0	0	0	0	0	1	0	0
Litter incidence	0	0	0	0	0	1	0	0
Filamentous tail								
Fetal incidence	1	0	0	0	0	0	1	0
Litter incidence	1	0	0	0	0	0	1	0
Total malformations								
Number with external malformations								
Fetal incidence	1	0	0	0	0	2	1	1
Litter incidence	1	0	0	0	0	2	1	1
Total number with malformations								
Fetal incidence	1	0	0	0	0	1	1	1
Litter incidence	1	0	0	0	0	1	1	1

fluid present. We observed this same lack of correlating statistical significance in our previous study (Carmines *et al.*, 2003) under similar exposure conditions.

In our previous studies, it was demonstrated that exposure of rats to reference cigarette smoke both before and during gestation resulted in lower fetal and birth weight (Carmines *et al.*, 2003; Gaworski *et al.*, 2004). In this study, it was demonstrated that exposure to cigarette smoke during the gestation period only is sufficient to produce reduced fetal weight. The study design was to terminate the dams and measure fetal weight 1 day prior to parturition. It was previously concluded that exposure to cigarette smoke reduced fetal weight as a result of maternal toxicity as indicated by the reduced maternal body weight gain (Carmines *et al.*, 2003). The current work with the individual smoke constituents does not support the hypothesis that the reduced maternal weight gain was responsible for the reduced fetal weight. On the contrary, it appears that the nicotine in the smoke may be principally responsible for the reduced maternal weight gain and that the CO is causing the reduced fetal weight. Exposure to nicotine vapor reduced maternal body weight gain but had essentially no effect on fetal weight. Conversely, exposure to CO had essentially no detrimental effect on maternal body weight gain but significantly reduced fetal weight. The mixture of CO and nicotine produced a pattern of reduced maternal body weight gain and also reduced fetal weight similar to that observed with cigarette smoke. Addition of a mixture of aldehydes had no effects. Based on this experimental work in rats, it appears that nicotine in smoke is responsible for the reduced maternal body weight gain, that CO is responsible for

reduced fetal weight, and that the two effects are not related. While the experimental exposure scenario and pharmacokinetics of the smoke and its individual constituents are likely different in rats and humans, this research suggests that CO could be a significant factor in the observed lower birth weight in humans. It is not possible to measure the day-to-day effect of smoking on human fetal weight development. The significance of this research is that under well-controlled conditions using an experimental model designed to mimic cigarette smoke exposure, it was demonstrated that nicotine had no effect on fetal weight and that CO appeared to be almost solely responsible for the reduced fetal weight in rats.

ACKNOWLEDGMENTS

The authors thank Richard Carchman, Chris Coggins, and D. E. Rodwell for their contributions to the preparation of the manuscript and the staff at IIT Research Institute for their excellent technical assistance.

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