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Nicotine- or epinephrine-induced uteroplacental vasoconstriction and fetal growth in the rat

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Abstract

We examined the relationship between nicotine-induced vasoconstriction in pregnant rat dams and fetal growth during the third trimester of pregnancy. Pregnant rats were continuously treated between days 13 and 19 of gestation with either nicotine (9.6, 4.8 or 2.4 mg/kg/day), epinephrine (0.72 μg/kg/day), or saline via continuous infusion from a subcutaneously implanted osmotic minipump. Placental weights in rats treated with high dose nicotine and dams' body weights were severely reduced. However, fetal weights were not affected. Blood flows in uterus and placenta were quantified by measurement of tissue content of ^{85}Sr-labelled microspheres injected via a carotid artery catheter. Both nicotine and epinephrine caused a significant reduction (> 40%) in uterine and placental blood flow. We conclude that vasoconstriction alone as a result of nicotine or epinephrine administration during the last trimester of gestation does not necessarily reduce nutrient supply to the fetus and does not affect fetal growth in rats.

Keywords: Cigarette smoke; Nicotine; Fetal growth; Blood flow; Osmotic minipump delivery; Epinephrine; Transplacental diffusion; Fetal nutrient supply

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1. Introduction

The potential health effects of exposure of individuals to environmental tobacco smoke (ETS; 'passive smoking') are controversial (U.S. Surgeon General, 1986; Reasor and Will, 1991). Even more controversial are suggested adverse effects on the fetuses of mothers exposed to ETS during pregnancy (U.S. Surgeon General, 1979; Rubin et al., 1986; Martin and Bracken, 1986; Trichopoulus, 1986; MacArthur and Knox, 1987). Due to inherent uncertainties in estimation of ETS dose to individuals and the ubiquitous presence of confounding variables in epidemiological studies, there is the necessity of developing reliable animal models of human exposure to ETS to resolve these issues and to allow for a critical examination of the underlying mechanisms were such effects to be observed. Over the past few years we have been attempting to develop such a model in the rat (Rajini et al., 1994; Witschi et al., 1994).

We have previously shown (Rajini et al., 1994) that exposure of pregnant rats to sidestream cigarette smoke (SS, a laboratory surrogate for ETS) on days 3, 6–10 and 13–17 of gestation causes small, but significant reductions in mean weight of offspring. Several possible mechanisms for such an effect of cigarette smoke have been suggested by others, including fetal hypoxia due to carbon monoxide and chronic reduced placental blood flow due to nicotine-induced vasoconstriction. The underlying pathogenic mechanism for nicotine described by most investigators is a constriction of the uteroplacental arteries, mediated through the release of epinephrine by the nicotine, causing reduced inflow of maternal blood into the placenta and reduced placental nutrient transfer (Resnik et al., 1979; Philipp et al., 1984; Nash and Persaud, 1988). Since the vasoactive effect of nicotine is mediated by the release of epinephrine, one would expect that the effects of nicotine and epinephrine on fetal development would be similar. However, data published by Trend and Bruce (1989) indicate that a seven-fold elevation of plasma epinephrine levels in pregnant rats does not affect fetal development, suggesting that vasoconstriction alone induced by epinephrine does not cause fetal growth retardation. Thus, some other nicotine-mediated mechanisms or other bioactive compounds of tobacco smoke may be responsible for the changes in fetal development. Unilateral ligation of the uterine artery in the early stages of gestation in the rat, a more drastic intervention in uterine blood flow, also has little effect on fetal development (Bar and Brent, 1970; Bruce, 1976). Thus the question of whether ETS-induced inhibition of intrauterine fetal development is indeed caused by nicotine-mediated vasoconstriction is certainly an unresolved issue.

In the present paper we attempted to examine this question directly by examining the relationship between nicotine induced-vasoconstriction in pregnant rats and fetal growth during the third trimester of pregnancy.

2. Materials and methods

2.1. Animals

Timed-pregnant rats (breeding day = day 0) of the Sprague–Dawley strain (mean body weight 217.3 ± 3.3 g) were obtained on day 12 of pregnancy from Charles

River Laboratories (Boston, MA, USA), and divided into five experimental groups, as delineated in Table 1. The group size was deliberately kept small to allow for the intense physiological analysis performed on each individual animal in the study. Each rat was housed individually in a stainless steel, wire bottom cage located in an environmentally controlled chamber with a temperature range of 23–25°C. Food and water were freely available throughout the experiment. Each rat received under light methoxyflurane anesthesia an Alzet minipump implanted subcutaneously (Model 2ML1, Alza Co., Palo Alto, CA, USA) on day 13 of gestation. The minipump was placed in a subcutaneous pocket made over the dorsal scapular region. Each rat in Group 1 was implanted with a minipump containing 0.85% NaCl and served as the controls. Rats in Groups 2, 3 and 4 were implanted with minipumps containing 100, 50 and 25 mg/ml of nicotine (free base form), to deliver a dose rate of 9.6, 4.8 and 2.4 mg/kg/day, respectively. These dose rates were chosen to give nominal plasma levels of about 140, 70 and 35 ng/ml, respectively (Murrin, 1987), values chosen to correspond to those used in our earlier study (Witschi, et al., 1994), using a very different method to deliver the nicotine to the animals. Group 5 rats were implanted with minipumps containing 7.5 mg/ml of epinephrine (adrenaline acid tartrate, Sigma Co., St. Louis, MO, USA) dissolved in a carrier solution containing ascorbic acid to inhibit oxidation (0.55 g of sodium chloride + 0.1 g of ascorbic acid in 100 ml of distilled water). Minipumps delivered 0.72 mg/kg/day of epinephrine for the rats in Group 5.

2.2. Regional blood flow measurements

The reference sample microsphere method as described by Ishise et al. (1980), with some modifications, was applied for blood flow measurements. On day 19 of gestation, all rats were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneally). A polyethylene catheter (PE-50) was inserted into the femoral artery. A second catheter of PE-10 polyethylene tubing was inserted into the right carotid artery and advanced 4–5 cm into the left ventricle. Placement of the catheter in the ventricle was confirmed by left ventricular pressure pulse tracing and during dissection. Both catheters were filled with heparin (20 IU/ml) in 0.85% NaCl to prevent clotting.

The femoral and carotid cannulae were connected to a Statham P-23-Db pressure transducer and a Graph-tech WR3600 recorder (Irvine, CA) for blood pressure measurements.

Radionuclide (^{85}Sr)-labelled microspheres (10 mCi/g, Dupont, Wilmington, DE, USA) 15 ± 3 μm in diameter were suspended in 10% Dextran solution with 0.01% Tween 80 added to prevent aggregation. The resulting suspension was used for measurement of regional blood flows. The femoral artery catheter was connected to a Harvard constant flow pump and arterial reference blood sample was collected at a rate of 0.4 ml/min. Approximately 400 000 microspheres labelled with ^{85}Sr in 0.2 ml of suspension were flushed into the left ventricle with 0.5 ml of saline over 20 s. Collection of the arterial blood sample was begun 5 s before injection of microspheres and continued for two min to ensure that all microspheres in transit in the peripheral arterial blood were collected.

Five minutes later, 60 μCi of technetium (^{99m}Tc) as a water soluble pertechnate

salt was injected via the carotid cannula. Three minutes later, two additional blood samples were collected from the femoral cannula for technetium counting and for analysis of plasma nicotine concentration. The dams were then euthanized with an intravenous overdose of sodium pentobarbital according to approved protocol procedures. The fetuses and placentas were carefully removed from the uterus and separated from the amniotic membranes and fluid. Maternal heart and kidneys, and samples of liver, muscle and uterus were also collected and blotted dry. Each tissue or blood sample was placed in an individual polystyrene 16 × 125 mm preweighed tube for gamma-counting, weighed, and counted at an efficiency of about 80% in a gamma counter (Packard Instruments Inc., Downer's Grove, IL, USA). Additional blood samples were collected as frozen plasma for nicotine and cotinine determination. Tissue blood flow was calculated as follows:

$$\frac{\text{cpm of } {}^{85}\text{Sr in organ} \times 100 \times \text{reference blood sample rate (ml/min)}}{\text{cpm of } {}^{85}\text{Sr in arterial reference blood sample} \times \text{tissue weight (grams)}} = \text{blood flow (ml/min/100 gr)}$$

The blood flow measurements in placentas, fetuses, heart, kidney, liver, muscle and uterus of the treated groups were compared to the values measured in the control group.

2.3. Tissue and maternal weights

The net weight gain of the dam between days 12 and 19 of gestation was calculated by the following equation: [dam weight day 19–(fetal+placental weights)]–dam weight day 12 = net weight gain. Placental and fetal weights were measured on day 19 of gestation, as were heart and kidney weights.

2.4. Transplacental diffusion rates

Transplacental diffusion rate changes were measured by comparison of the relative percentages of technetium found in the fetuses versus the placentas of the different experimental groups. The data are calculated as the percentage of counts present in the fetus to the total counts in the conceptus (placenta + fetus) per g of tissue using the formula:

$$\frac{\text{counts/min per g of fetus}}{\text{counts/min per g of fetus} + \text{counts/min per g of placenta}} \times 100$$

Only fetuses that had vital signs after removal from the uterus were included in the experimental data analyzed.

2.5. Plasma nicotine and cotinine assay

Plasma nicotine and cotinine concentrations were determined by gas chromatography with use of a nitrogen-phosphorus detector. This technique is a modifica-

tion of a previously published method (Jacob et al., 1981), which allows simultaneous extraction of nicotine and cotinine and use of a capillary gas chromatograph. Briefly, 1 ml plasma samples were spiked with two internal standards (*S*-methylnicotine and 1-methyl-*S*-(2-pyridyl)-pyrrolidin-2-one *ortho*-cotinine) at concentrations near the expected average sample concentrations for nicotine and cotinine, respectively. To this was added 0.5 ml of 2 N NaOH: 0.2 M NH_4OH and 3 ml of toluene:butanol (70:30). The mixture was then mixed vigorously with a Vortex mixer (3 min), centrifuged (311 $g \times 5$ min), and chilled in an acetone/dry ice bath to freeze the aqueous layer. After the aqueous phase was discarded, 0.5 ml of 1 N H_2SO_4 was added to the organic phase and again the samples were mixed, centrifuged and frozen. After the organic phase was discarded, 0.5 ml of 50% K_2CO_3 and 0.4 ml of toluene:butanol (90:10) was added to the aqueous phase, and again the samples were vortex mixed, centrifuged and frozen. The organic phase was retained and a two microliter aliquot was injected onto a gas chromatograph for analysis. Nicotine and cotinine plasma concentrations were determined by the peak area ratio between either nicotine or cotinine and their respective internal standards. The gas chromatograph used was a Hewlett Packard Model 5890 (Hewlett-Packard Company, Avondale, PA) with a nitrogen-phosphorus detector. Data were recorded and analyzed with a Hewlett Packard 3393A integrator (Hewlett-Packard Company, Avondale, PA) A DB-5 capillary megabore column, 30 m × 0.53 mm I.D. (J & W Scientific, Folsom, CA) was used in the splitless mode with a helium linear flow velocity of 18 cm/s and a N_2 makeup gas flow of 19.9 ml/min. Injection port and detector temperatures were 250°C and 300°C, respectively. Oven temperature was controlled by a gradient program with an initial temperature of 90°C with a 0.5 min hold, followed by a 25°C/min increase with a 3 min hold after 250°C was reached. A four point standard curve was constructed by spiking rat plasma with the two internal standards at the same concentrations used for the unknown samples plus incrementally increasing concentrations of nicotine and cotinine. Results are reported as ng (free base form) per ml of plasma. Nicotine bitartrate and cotinine (free-base form) were purchased from Sigma Chemical Co., St. Louis, MO. *S*-methylnicotine bis-oxalate and *ortho*-cotinine perchlorate were both gifts from Peyton Jacob, III, Ph.D., Division of Clinical Pharmacology, Department of Medicine, University of California, San Francisco.

2.6. Data analyses

All results presented in this paper are mean values ± 1 S.D. The mean fetal weights, placental weights, placental blood flows and transplacental diffusion rates were evaluated for each treatment group by two different statistical approaches. Each treated group was either treated as a uniform unit resulting in a single mean of all fetuses per experimental group, or each litter within each treatment group was treated as a single unit where the number of samples (N) per group was the number of litters. Group mean values were compared with the control group using a computerized Generalized Linear Model (GLM) for analysis of variance (ANOVA), followed by a t-test. A value of $P < 0.05$ was taken to indicate significance. The mean values of blood flows in heart, liver, muscle and uterus in the dams of the treated groups were compared to the mean values of the control group as described above.

3. Results

Five groups of pregnant rats were studied on day 19 of gestation (cf. Table 1). The average body weight gain in the control and epinephrine-treated rats was found to be 46 ± 11 and 50 g (> 20% of the starting body weight), while the high dose (9.6 mg/kg/day) nicotine-treated rats lost 3 ± 1 g (to 214 g maternal body weight). The medium dose group (4.8 mg/kg/day) gained 15 ± 6 g of body weight, while the low dose group gained 34 ± 7 g during gestation, clearly indicating a negative dose response between maternal weight gain and nicotine administered. Fetal weights, however, were in the range of 2.3–2.5 g in all of the groups studied; no significant difference in fetal weight was observed for any of the experimental groups.

Placental weights in the high dose nicotine-treated rats were apparently decreased by about 15% as compared with the control group (0.49 ± 0.08 vs. 0.58 ± 0.13 g, respectively). This difference was not statistically significant. The low dose nicotine-treated rats, on the other hand, had significantly increased (about 23%) placental weights, of 0.75 ± 0.11 g. No effect on placental weight was found in medium dose nicotine- (0.61 ± 0.11 g) or epinephrine- (0.62 ± 0.13 g) treated rats.

Placental blood flows, as quantified by the radioactive microsphere technique, were significantly decreased in the rats treated with either nicotine or epinephrine, to 45–56% of the control value of 160 ± 12 ml/min/100 g. The differences between the treated and control groups were highly significant ($P < 0.01$). Actual values found for the low, medium, and high dose nicotine- and the epinephrine-treated groups were 63 ± 5, 72 ± 6, 93 ± 23 and 71 ± 11 ml/min/100 g, respectively (Fig. 1); the data for the treated animals indicated a (non-significant) trend towards a dose-related response.

Uterine blood flows were also quantified by the radioactive microsphere technique, as shown in Fig. 2. Flow rates were significantly reduced in all nicotine-treated rats when compared to the control group values of 96 ± 9 ml/min/100 g. The rats treated with high dose nicotine had a blood flow of 64 ± 7 ml/min/100 g, the medium dose group 42 ± 10 ml/min/100 g, and the low dose group 69 ± 15 ml/min/100 g; the epinephrine-treated group had a flow rate of 45 ± 16 ml/min/100 g. Several other maternal organs were examined. Heart weights were found to be significantly lower only in the high dose nicotine group (0.61 ± 0.06 g) when com-

Table 1
Experimental Groups

Group no.	No. of rats in group	Treatment (mg/kg/day)
1	6	Saline (0.85% NaCl)
2	10	Nicotine, 9.6
3	4	Nicotine, 4.8
4	4	Nicotine, 2.4
5	5	Epinephrine, 0.72

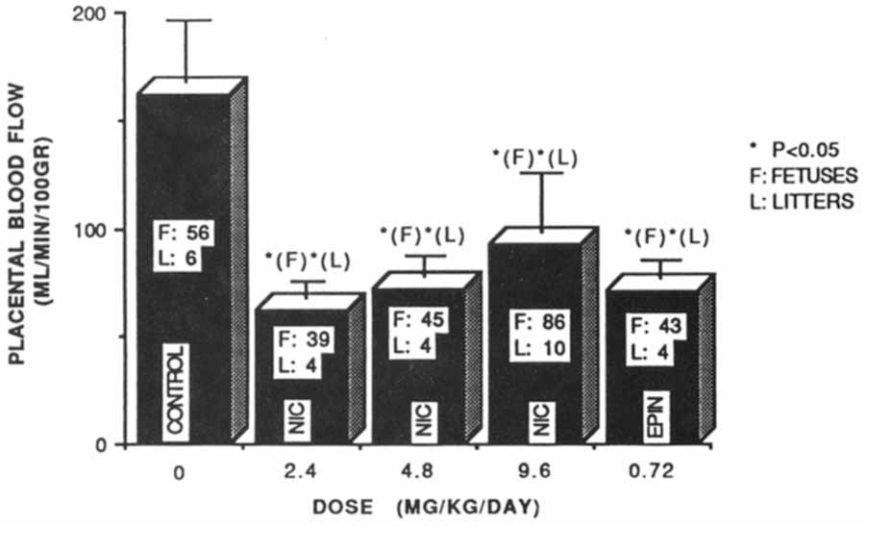

Fig. 1. Placental blood flow in nicotine- or epinephrine-treated rats. The rats were treated by continuous delivery of the drug from an Alzet osmotic minipump starting on day 14 of gestation. The placental blood flow was measured on day 21 of gestation. Data are presented as the mean ± 1 S.D. of the measured blood flows per treatment group. NIC: nicotine. EPIN: epinephrine.

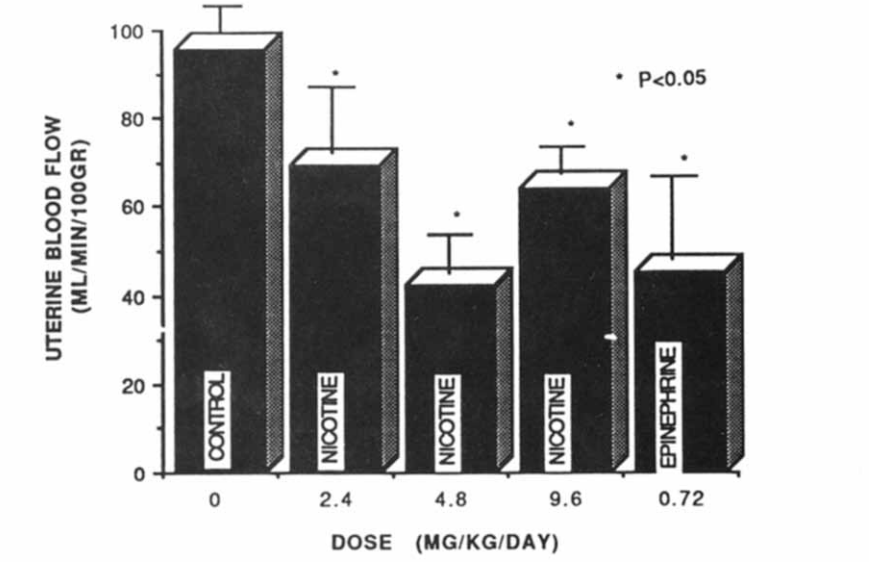

Fig. 2. Uterine blood flow in nicotine- or epinephrine-treated rats. The rats were treated by continuous delivery of the drug from an Alzet osmotic minipump starting on day 14 of gestation. The uterine blood flow was measured on day 21 of gestation. Data are presented as the mean ± 1 S.D. of the measured maternal blood flows per treatment group.

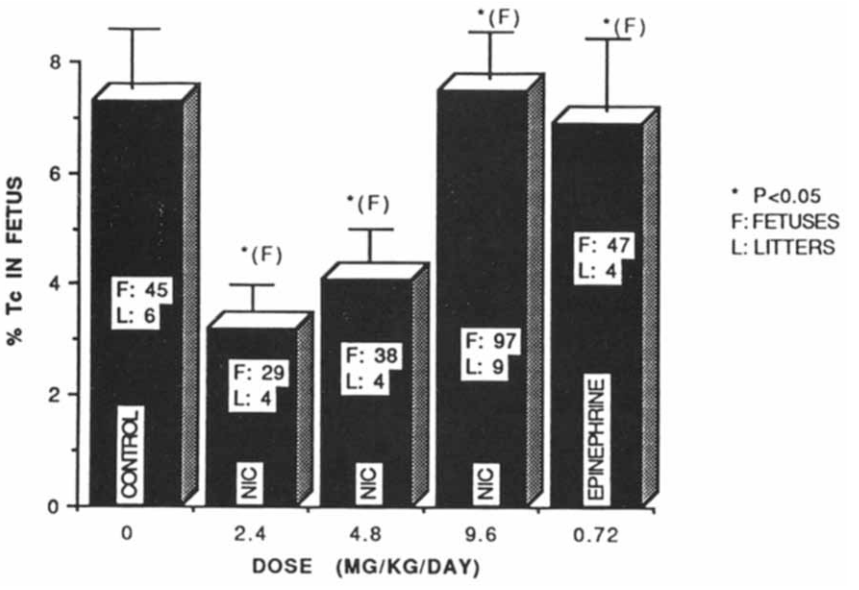

Fig. 3. Transplacental diffusion in nicotine- or epinephrine-treated rats. The low molecular weight tracer (^{99m}Tc as a pertechnate salt) was injected on day 21 of gestation. The relative amount of tracer found in the fetus is expressed as a percent of the total amount in the conceptus.

pared to the control group (0.85 ± 0.07 g). Kidney weights in the high dose nicotine group were also significantly reduced, 0.77 ± 0.13 g versus 0.98 ± 0.18 g in control rats. Blood flows in kidneys of all treated groups were in the range of 254–262 ml/min/100 g. The kidney blood flow for epinephrine-treated rats trended towards higher values (298 ml/min/100 g), but the observed difference was not statistically significant.

We observed an apparent trend towards an increased delivery of blood-borne nutrients to the fetuses of mothers given high-dose nicotine or epinephrine when the diffusion rate of soluble, low molecular weight ^{99m}Tc radiotracer from the maternal blood to the fetus was examined. The amount of technetium in fetuses of the high dose nicotine-treated group was elevated by 7.5% while the amount of technetium was increased by 8.4% in the epinephrine-treated group. A decrease in the transplacental diffusion of technetium was found in the medium and low dose nicotine-treated groups, 4.2 and 3.2%, respectively, as compared to the control group (5.8%). These differences were significant only when evaluated per fetus; they were not significant on a per litter basis (Fig. 3). However, there might have been a systematic bias in our results had the relative amount of ^{99m}Tc in the fetus been a function of the amount of ^{99m}Tc in the placenta, since placental blood flow was decreased in the nicotine- and the epinephrine-treated rats (cf. Fig. 1). Thus, we examined whether there was a direct correlation between placental concentration of ^{99m}Tc and fetal levels by linear regression analysis, as shown in Fig. 4. We found a

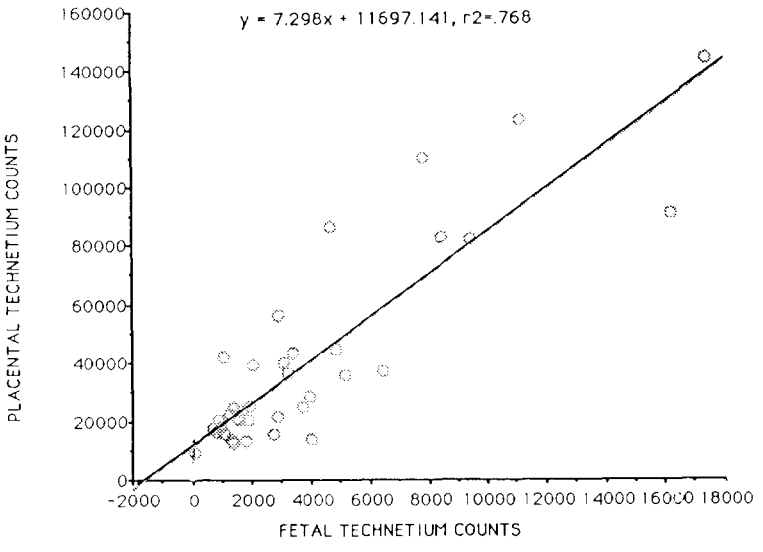

Fig. 4. Regression line of counts/min in placenta versus counts/min in fetus. See text for details.

correlation coefficient between placental and fetal levels of ^{99m}Tc of $r^2 = 0.77$, indicating that the relative amount of technetium in the fetus was directly proportional to the technetium concentration in the placenta (maternal blood). Thus, even though absolute amounts of ^{99m}Tc were higher in the placentas of the control rats, the relative amount diffusing to the fetus was well described by the same first-order rate equation for diffusion over the entire range of placental concentrations encountered in this study (about a 15-fold range of ^{99m}Tc counts in the placental blood).

3.1. Plasma nicotine concentrations

Plasma nicotine concentrations were quantified for the various groups of rats examined in this study at the time of sacrifice, as measured by gas chromatography. The average nicotine concentration found in the group treated with 2.4 mg/kg/day was 37 ± 25 ng/ml, in the 4.8 mg/kg/day group it was 117 ± 25 ng/ml, and in the 9.6 mg/kg/day nicotine group it was 382 ± 80 ng/ml. Cotinine levels were 200 ± 80, 500 ± 102 and 1080 ± 200 ng/ml, respectively, in these animals.

4. Discussion

In this study we attempted to directly test the hypothesis that the ETS-induced retardation of intrauterine fetal growth that we observed in our earlier studies (Rajini et al., 1994) could directly be attributed to nicotine-mediated vasoconstriction of the uteroplacental arteries. To do this, we administered constant infusions of nicotine to pregnant rats in the third trimester of pregnancy. We chose to use nominal

concentrations of nicotine of 100, 50 and 25 mg/ml in the minipump experiments, to deliver an approximate dose rate of 9.6, 4.8 or 2.4 mg/kg/day, respectively. According to previously published data (Murrin et al., 1987), these dose delivery rates should have achieved plasma nicotine concentrations of about 40–160 ng/ml at steady state in non-pregnant animals. However, we found higher plasma concentrations of nicotine, namely 380 and 117 ng/ml in the high and medium dose-treated groups, respectively. Humans smoking high nicotine cigarettes reportedly had steady-state concentrations of nicotine that averaged 29–30 ng/ml of plasma (Russel et al., 1976). Thus, the rats in our study were exposed to concentrations of nicotine that ranged from values similar to those reported for human exposures (low dose group) to about 13-fold higher (high dose group). However, humans metabolize nicotine more rapidly than do rats, so direct comparison of blood levels may be misleading. In addition, our rats had continuous exposure to steady-state levels of nicotine rather than short peaks while smoking.

The results of the present study show that nicotine at a concentration in plasma (380 ng/ml) much higher than that found in heavy smokers prevents the pregnant dam from gaining weight, and also results in decreased weight of vital organs like heart and kidney. However, fetal weight is not affected in these animals. It is interesting to note that only one rat in the high dose nicotine group, with a unilateral pregnancy (not included in our data), gained weight in spite of the nicotine treatment, indicating that maternal weight gain may only be possible after all nutritional needs of the fetuses are fulfilled. Pregnant rats in the medium and low dose nicotine groups gained weight in this study, but significantly less than the controls, indicating that administration of nicotine to rats between days 13 and 19 of gestation at concentrations high enough to produce plasma levels comparable to those measured in heavy smokers, or as much as 13 times higher, had a strong inhibitory effect on maternal weight gain, but no effect on fetal weight. These findings are even more noteworthy since parallel measurements in the same rats show a substantial reduction in uteroplacental blood flow in the nicotine-treated rats. The reduction of blood flow seen in these rats agrees with observations reported for Rhesus monkeys (Suzuki et al., 1974) and for sheep (Monheit et al., 1983; Resnik et al., 1979) administered nicotine.

Epinephrine administration in our experiments did not affect the weight gain of the dam or of the fetus, but it caused a similar reduction in uteroplacental blood flow as was seen in the nicotine-treated rats. Epinephrine treatment during all three weeks of gestation also reportedly did not affect fetal growth (Trend and Bruce, 1989). It has been suggested that reduced birth weight of infants whose mothers smoked during pregnancy is due to impairment of blood flow to the placenta, caused primarily by the vasoconstrictive effect of nicotine as mediated by epinephrine (Nash and Persaud, 1988). Our experimental data, however, do not support this assumption. During the last 7 days of gestation (the period we treated our rats includes 5 of these days), fetal growth in the rat is over seven times faster than during the first 14 days of gestation (Hebel and Stromberg, 1986). It is, therefore, surprising that while fetal nutrient demand was highest in these animals a uteroplacental blood flow reduction of over 50% did not affect fetal growth. Therefore, we conclude that vasoconstriction alone does not cause fetal growth retardation and that there are probably effective

compensatory mechanisms to prevent a nutrient shortage to the fetus when uterine vasoconstriction occurs. This is even more important when we consider the fact that the uteroplacental vascular system is known to be more responsive to vasoactive drugs than other organs, as our experimental results also indicate. We did not observe blood flow changes in heart, kidney, liver, or muscle, while the measured reduction in uterus and placental flow was more than 40–50%.

The compensatory capabilities for blood flow alterations in the rat are efficient enough to prevent fetal growth retardation even after a unilateral ligation of the uterine artery performed at an early stage of gestation (Bar and Brent, 1970; Bruce, 1976, 1978). A possible compensatory mechanism that has been suggested in humans is the relative increased rate of growth of the placenta in smoking mothers (Kullander and Kaellen, 1971). Our experimental results show a trend toward increased placental weight in rats treated with low dosage of nicotine, no effect in the medium dose group, but a significant decrease of placental weight in the rats treated with the high dose of nicotine, suggesting that there may be a correlation between physiological placental changes in response to nicotine administration, which are reflected by changes in weight, and the measured transplacental diffusion rate.

In conclusion, our data show that uteroplacental vasoconstriction, whether induced by epinephrine or nicotine at plasma levels either similar to those found in heavy smokers or substantially higher, does not affect fetal growth in the rat. While fetal growth retardation observed in rats exposed to SS is probably not caused by decreased blood supply to the placenta due to epinephrine-induced uterine vasoconstriction secondary to nicotine ingestion, the fetus might respond directly to bioactive tobacco smoke components. The complex composition of tobacco smoke makes it exceedingly difficult to relate the pharmacological, physiological and morphological consequences of tobacco consumption during pregnancy to the actions of individual compounds in the smoke. Thus, the mechanism(s) underlying the observation that SS produces intrauterine growth retardation in the rat needs further investigation.

Acknowledgments

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