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Gestational exposure to nicotine in drinking water: teratogenic effects and methodological issues

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Abstract

Individual differences in nicotine effects lead to questions about appropriate experimental procedures for prenatal nicotine exposure in rodent models. The objective of this study was to develop a method for gestational studies in rats based on oral nicotine exposure, and to evaluate the neurodevelopmental effects. Female Lister hooded rats were exposed to nicotine solutions both before and during pregnancy. These females were divided into groups consuming solutions of different concentrations such that animals that initially consumed the solutions most readily were exposed to progressively higher concentrations. Offspring of these females were evaluated in a test battery measuring maturational and developmental milestones. Female rats ingested nicotine solutions at levels that provided blood nicotine concentrations of 10–60 ng/ml, at daily dose levels of 2.9–6.2 mg/kg. Solutions with concentrations below 0.06 mg/ml were well tolerated with some moderate adverse effects at the highest dose. Concentrations above 0.08 mg/ml led to a large drop in fluid consumption and body weight. Strong teratogenic effects of prenatal nicotine exposure were observed at concentrations above 0.04 mg/ml, including developmental and maturational delays shown by measures of pinnae detachment, fur appearance, incisor eruption, eye opening and righting reflex. Negative geotaxis, grip strength and weight gain were impaired and postnatal mortality was increased. This study design provides a model for the impact of prenatal exposure to nicotine at blood levels comparable with those in medium and heavy smokers. There were marked developmental and behavioural deficits induced in the offspring of nicotine-exposed female rats.

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Keywords

nicotine; rat; pregnancy; teratogenic effects; neurodevelopment; behaviour

Introduction

Cigarette smoking is associated with a wide variety of adverse reproductive outcomes (Jauniaux et al., 2007). Children born to women who smoked during pregnancy are more likely to be born preterm, have a low birth weight and have an increased risk of sudden infant death syndrome (Ernst et al., 2001; Winzer-Serhan, 2008). Prenatal exposure to nicotine may also lead to disturbed neurodevelopment and acceleration of the risk for a range of psychiatric problems, including ADHD-related disorders and substance abuse (Pauly and Slotkin, 2008). A major teratogenic component of tobacco smoke responsible for adverse neurodevelopmental effects is nicotine, which, via activation of nicotinic acetylcholine receptors (nAChRs), modulates neurotransmitter release, gene expression, neuronal outgrowth, cell survival, and synapse formation and maturation (Dwyer et al., 2008).

A typical smoker using 20 cigarettes a day will absorb about 0.3 mg/kg nicotine daily, resulting in peak plasma nicotine concentrations in the range of 10 to 50 ng/ml (Benowitz and Jacob, 1997). These parameters might be much higher in heavy smokers, but individual differences in nicotine kinetics and metabolism are common (Benowitz and Jacob, 1997) and are also seen in susceptibility to nicotine addiction and the development of nicotine-related health problems (Shiffman and Paton, 1999). It has been suggested that vulnerability to nicotine dependence might relate to high initial sensitivity to nicotine combined with more rapid development of tolerance, and subsequently higher self-administration (Pomerleau, 1995); however the basis for such individual responses to nicotine is not yet well understood.

Individual variation in responses to nicotine raises questions about the best experimental design for nicotine exposure; for example, whether a fixed dose or subject-determined doses should be administered. Recently, Rose et al. (2003) proposed a procedure in which nicotine dose delivered during experiments in humans was based on nicotine intake per puff during a baseline unlimited access smoking session, providing each smoker with an individually comfortable level of nicotine. A similar approach can be taken in animal studies and was adopted in the present study. Importantly for cross-species comparisons, account must be taken of significant differences in metabolism of nicotine in humans and rodents. Plasma nicotine $t_{1/2}$ in rats is shorter than in humans (45 minutes vs. 2 hours respectively), necessitating the use of higher daily doses of nicotine in rats to achieve blood nicotine concentrations that are comparable to those seen in human smokers (Seaton and Vesell, 1993; Matta et al., 2007).

The most commonly used method for chronic nicotine exposure in rats is the 's.c. osmotic minipump'. In gestational nicotine exposure studies, the use of minipumps eliminates the foetal hypoxic consequence of utero-placental vasoconstriction resulting from repeated exposure to nicotine via other routes (Matta et al., 2007), but the procedure has several inherent problems. First, this mode of delivery results in a chronic level of nicotine, in contrast to the pulsating mode of delivery via smoking. This may have functional significance, as chronic continuous nicotine exposure results in desensitization of nAChRs, whereas during episodic smoking nAChR function fluctuates, with periods of activation-desensitization-resensitization, which is considered an important factor in the addictive properties of nicotine (Benowitz, 1999) and its effects on the foetus (Jauniaux et al., 2007).

Second, rodents usually increase their weight over the course of these experiments, especially pregnant females that can gain over 50% of their initial weight. This might be a particular problem when lower doses of nicotine are used, as the effectively delivered dose will inevitably decline during the study.

To model more closely the chronic exposure experienced by habitual smokers, nicotine in drinking water was used in the present study. Several laboratories have reported that rats will ingest nicotine in their drinking water at levels that provide nicotine intake comparable to the nicotine received by humans smoking cigarettes (Peters and Tang, 1982; Maehler et al. 2000). The same phenomenon has been shown in pregnant females (Peters and Tang, 1982; Paz et al., 2007); however, in some studies oral nicotine in female rats has led to a serious drop in fluid consumption and problems with weight gain (Murrin et al., 1987). This might have been induced by activation of taste pathways and low palatability of nicotine (Simons et al., 2006), frequently requiring pre-training nicotine habituation periods (Maehler et al., 2000) or use of the sucrose-fading method (Smith and Roberts, 1995). Nevertheless, this oral approach has the advantage that treatment is episodic, as it occurs only when animals drink, and is relatively stress-free. The caveat is that the precise control of nicotine intake is limited by the individual pattern of fluid intake and individual tolerance of the nicotine concentration, which can lead to large variances of the mean consumption and teratogenic effects between groups of animal.

Common physical and behavioural effects of prenatal exposure to nicotine have been observed in previous studies including, intolerance to hypoxia (Slotkin et al., 1995), persistent alterations in cholinergic (Gold et al., 2009) and catecholaminergic neurotransmitter systems (Oliff and Gallardo, 1999), hyperactivity (Tizabi et al., 1997; Pauly et al., 2004), cognitive impairments (Levin et al., 1993; Vaglenova et al., 2008), increased anxiety (Vaglenova et al., 2004; Sobrian et al., 2003), and somatosensory deficits (Abou-Donia et al., 2006). However, these findings are not entirely consistent since some studies found no differences in locomotor activity (Martin and Becker, 1970) and cognitive performance (Bertolini et al., 1982; Paulson et al., 1993), as well as hypoactivity (LeSage et al., 2006). Some of the reported neurobehavioral outcomes are gender specific, with overall a greater impact in males (Peters and Tang, 1982; Pauly et al., 2004; Vaglenova et al., 2008). Prenatal nicotine exposure in rodents has also been related to developmental delay and maturational deficits including, lower birth weight, delay in eye opening, compromised righting reflex and negative geotaxis, with results varying as a function of nicotine dose, schedule and route of administration (Peters and Ngan, 1982; Peters and Tang, 1982; Ajarem and Ahmad, 1998; Murrin et al., 1987; Paulson et al., 1993, 1994; Paz et al., 2007; Vaglenova et al., 2008).

The first goal of the study reported here was to modify and evaluate the oral method, of which the potential value was not brought out fully by previous reports. It is well suited for investigations involving prolonged exposure and growing animals and therefore we aimed to make it a viable experimental approach to model prenatal exposure to nicotine in the rat. The second goal was to evaluate neurodevelopmental effects of prenatal exposure to different doses of nicotine to find an optimal nicotine dose for the gestational studies. To this aim, female rats were exposed to nicotine solution as the only source of water both before and during pregnancy and their offspring evaluated in a battery of maturational and developmental tests.

Methods

Study design

Optimal doses were chosen on the basis of nicotine plasma levels obtained in the pilot experiment described below. Then, in the main experiment, neurodevelopmental effects of prenatal exposure to different doses of nicotine were studied in male and female offspring. In each experiment females were divided into groups consuming different concentrations of nicotine solution on the basis of their actual consumption/tolerance to nicotine effects. In addition, a cross-fostering procedure was included to avoid potential negative effects of nicotine exposure and withdrawal on the rearing and consequently on neonatal development and behaviour of the offspring. We expected that this procedure for nicotine exposure would diminish within group variability in nicotine solution consumption introduced by the individual pattern of fluid intake/nicotine tolerance, leading to more consistent dose-dependent effects on maturational and developmental milestones in offspring.

Subjects

Both male and female Lister hooded rats (Harlan Olac, Bicester, UK), were used. They were housed individually (except during mating) and had free access to food and drinking fluids (tap water or nicotine solutions). National and institutional guidelines for housing and treatment were followed. They were maintained in a temperature-controlled environment (21 ± 1 °C) at 50% humidity and on a 12-h light/dark cycle.

Drug

Nicotine bitartrate (Sigma, USA) was dissolved in the drinking water at varying doses. Nicotine-containing water was adjusted to the pH of drinking water with 0.001 N NaOH. Doses are always presented as those of nicotine base.

Pilot experiment

The doses of nicotine employed in the pilot experiment were based on previous data showing that exposure to nicotine in the range of 1–6 mg/kg/day results in plasma nicotine levels of approximately 21–75 ng/ml (Murrin et al., 1987; Benwell et al., 1995; Paz et al., 2007). This range of plasma concentrations corresponds reasonably well with plasma levels in habitual smokers, with the higher value at the upper limit for nicotine levels in heavy smokers (Benowitz et al., 1990). We assumed a 30% drop in solution consumption during exposure to nicotine and used doses from 0.3 to 1.2 mg/ml, aiming for daily nicotine consumption in the range of 2 to 7 mg/kg.

Twenty female rats weighing 175–195 g at the beginning of the study were used to assess blood nicotine levels. The animals were divided into two groups balanced according to body weight. The initial dose of nicotine during the first week was 0.03 mg/ml. In the second step (week 2) animals drinking nicotine solution were divided into two groups: NIC 0.03 mg/ml (n=5) and NIC 0.06 mg/ml (n=10) as follows: the one third of the animals that drank the smallest amount of the 0.03 mg/ml nicotine solution stayed on this concentration to the end of the experiment; the remaining two-thirds of the animals were moved to the 0.06 mg/ml dose. The group consuming the 0.06 mg/ml nicotine solution was divided into two groups (week 3): 0.06 mg/ml (n=5) and 0.12 mg/ml (n=5) according to the criterion: the half of the animals that drank the smallest amount of the 0.06 mg/ml nicotine solution stayed on this dose and the remaining animals were moved to the highest concentration (0.12 mg/ml). Animals on the 0.12 mg/ml concentration drank very small amount of the solution (30–40% of the control group) and were transferred to a 0.1 mg/ml concentration from week 4, after which all animals were killed. Animals were killed by decapitation and the fluid draining

from the trunks was collected. Plasma levels of nicotine were assayed by gas chromatography.

Main experiment

Sixty male rats weighing 245-263 g and 110 nulliparous female rats weighing 130-192 g at the beginning of the study were used. They were handled regularly during the first two weeks after arrival. On the last three days of handling the rats were weighed and the average weight was calculated for each rat. Animals were divided into three groups (nicotine exposure, control group and future foster mothers) balanced according to their body weight.

Habituation to nicotine solution—Habituation to increasing concentrations of nicotine solution in tap water as the only source of fluid was introduced in 46 females and lasted for four weeks. Sixty-four female rats continued to receive tap water for the duration of the experiment. During the first week the lowest concentration of 0.03 mg/ml was presented. From the second week animals were moved to the 0.04 mg/ml concentration. At the next step (week 3) the females drinking nicotine solutions were divided into two groups: NIC 0.04 mg/ml (n=16), NIC 0.06 mg/ml (n=30) according to the criterion: one third of the animals drinking the smallest amount of the 0.04 mg/ml nicotine solution stayed on this dose to the end of the experiment; the remaining two-thirds were moved to 0.06 mg/ml dose. During the fourth week of exposure females drinking NIC 0.06 mg/ml solution were divided into two groups: NIC 0.06 mg/ml (n=15) and NIC 0.08 mg/ml (n=15) as follows: the half of the animals drinking the smallest amount of the 0.06 mg/ml nicotine solution stayed on this concentration to the end of the experiment, the remaining rats were moved to 0.08 mg/ml concentration. The criterion for moving animals to the next concentration of nicotine was that the amount of the drug solution consumed was at least 70% of the water consumption in the control group. The final concentrations of nicotine used were 0.04, 0.06 and 0.08 mg/ml.

Mating—Females were controlled according to their oestrous cycle. Females in proestrus and oestrous were mated during the dark phase of the day at the beginning of the fifth week of nicotine exposure. Nicotine solution was not withheld before mating. The day on which a vaginal plug or spermatozoa in the vaginal smear were found was defined as gestational day 0.

Pregnancy—Pregnant females from nicotine and control groups were weighed twice weekly. Consumption of nicotine solution was assessed on a daily basis. Food consumption was evaluated twice weekly. Foster mothers (N=49) had no previous exposure to nicotine and were handled regularly twice a week.

Birth measures—All dams were checked twice daily (08.00-08.30 and 16.00-16.30 h starting a few days before delivery. Deliveries completed by 08.30 h were assigned to postnatal day 1 (PND1). Pups born later that day were assigned to PND1 on the following morning. Litters were examined on PND1 for obvious morphological anomalies (e.g. missing digits, facial malformations, etc.), sexed by relative ano-genital distance and, in the case of litters with more than eight offspring, culled randomly to eight pups with equal numbers of males and females whenever possible. Gestation length was calculated at birth and the following litter data were collected on PND1: litter size, sex ratio, body weight for each pup and the number of malformed offspring. Litters were cross-fostered and the pups were evaluated throughout the lactation period in terms of reflex development and neuromuscular maturation. Developmental and maturational measures were collected from twelve control litters (41 females, 54 males), five litters exposed to 0.04 mg/ml nicotine solution (15 females, 25 males), five litters exposed to 0.06 mg/ml nicotine solution (20

females, 13 males), and four litters exposed to 0.08 mg/ml (7 females and 5 males). Tests were selected from standard neurobehavioral developmental test batteries (Adams, 1986).

Developmental milestones—The dam was first removed from the home cage and specific tests measuring reflex development, motor coordination, muscle strength, and locomotor activity were applied to the offspring. The order of testing litters was pseudo-random on each day. All testing was conducted between 09.00 and 16.00 h.

To assess righting reflex each pup was given two successive trials per day from PND two to five, timed from being placed in a supine position until it righted itself onto all four feet. The cut off time was 30 s. Surface righting reflects the development of labyrinthine and body righting mechanisms as well as vestibular function and motor development.

Negative geotaxis was observed daily from PND7 to PND10; pups were timed for completing a 180° turn within 30 s when placed in a head-down position on a 25° inclined wooden surface. Rats were given two consecutive trials per day and the mean was calculated. It reflects vestibular function, motor development and activity.

Forelimb grip strength was assessed on PNDs 14 and 17; a steel wire (20 cm long, about 0.3 cm thick) was supported between two poles of wood 25 cm above the table covered with soft towels; the latency to fall off the wire grasped by both forepaws was measured with a maximum time of 20 s; it measures muscle strength.

Locomotor activity was assessed on PND19 in photocell activity cages measuring 30×30×30 cm, by counting the interruptions of two beams of infrared light during a single 30 min session. The ‘cage cross’ measure of ambulation was the number of movements from one beam of light to the other (Reavill and Stolerman 1990).

Maturation milestones—Pups from each litter were weighed on PND1, PND5, PND10, PND15, and PND20. The emergence of physical maturation landmarks were noted, including pinnae detachment (PND3–5), incisor eruption (PND7–11), fur appearance (PND9–10), and eye opening (PND12;). Eyes were recorded as open only when both eyes were open.

Statistical analysis

Maternal and litter characteristics were analyzed by one-factor ANOVAs with prenatal treatment as the factor. Offspring development, maturation and locomotor activity were analyzed using two- and three-way ANOVAs with prenatal treatment and sex as between-subjects factors, followed by Bonferroni modified Least Significant Difference test (LSD) for *post-hoc* analysis. The litter mean score was used as a unit for statistical analysis. For those variables assessed multiple times over the neonatal period, age was used as a repeated factor. The level of statistical significance was set at $p < 0.05$.

Results

Pilot experiment

Daily nicotine solution consumption during the last week of exposure was 92.9 ± 10.5 , 90.3 ± 9.8 , 63.6 ± 12.2 ml/kg in groups exposed to nicotine at 0.03, 0.06 and 0.1 mg/ml, respectively (means $\pm$ SD); this compares to 102.1 ± 7.4 ml/kg in control animals. The daily nicotine consumption was 2.79 ± 0.32 , 5.42 ± 0.58 , 6.36 ± 0.54 mg/kg in groups exposed to 0.03, 0.06 and 0.1 mg/ml, with corresponding nicotine blood levels of 10.7 ± 6.5 , 60.0 ± 31.5 , and 52.4 ± 23.9 ng/ml. A huge (70%) drop in solution consumption was observed during exposure to the 1.2 mg/ml concentration and there was a decrease of almost 40% at a

concentration of 0.1 mg/ml. For the main experiment three nicotine solutions were chosen: 0.04, 0.06 and 0.08 mg/ml, to obtain daily nicotine ingestion similar to that observed in heavy smokers (Benowitz et al. 1990).

Main experiment

Nicotine exposure before and during pregnancy—The characteristics of females exposed to nicotine before mating and during pregnancy are summarized in Table 1. Four weeks of pre-exposure to increasing doses of nicotine as the only source of water resulted in decreased body weight gain ($F(3,56)=42.9$, $p<0.001$) and lower body weight before mating ($F(3,56)=27.9$, $p<0.001$) in all exposed groups compared to control animals. During the last week of pre-exposure, when the final concentrations of nicotine solutions were used, solution consumption was decreased in all exposed groups ($F(3,56)=35.1$, $p<0.001$). Additionally, body weight before mating and solution consumption were decreased in the 0.08 mg/ml as compared with the 0.04 mg/ml group. Consequently, dose-dependent nicotine ingestion was observed ($F(2,42)=74.8$, $p<0.001$) with all exposed groups differing significantly from each other. The effects of nicotine on food consumption in the last week of pre-exposure were not as dramatic as the effects on water consumption, but overall, they were statistically significant ($F(3,56)=3.18$, $p<0.03$).

Nicotine exposure during pregnancy led to significant differences in body weight gain among groups ($F(3,42)=9.02$, $p<0.001$) with groups 0.06 and 0.08 mg/ml gaining less weight than control animals, and with the 0.08 group significantly below the 0.04 group. Differences in nicotine solution consumption among groups ($F(3,42)=93.9$, $p<0.001$) were a result of diminished solution consumption in all exposed groups as compared with control animals. Additionally, the 0.08 mg/ml group consumed significantly less solution than the 0.04 mg/ml group. Consequently, dose-dependent nicotine ingestion was observed ($F(2,31)=60.5$, $p<0.001$) with all exposed groups differing significantly from each other. Similar to what was observed during the pre-exposure period, the effects of nicotine on food consumption were less dramatic than the effects on water consumption, but overall they were significant ($F(3,42)=3.68$, $p<0.02$), as a result of a decreased food consumption in the 0.08 mg/ml group compared to all other animals. Interestingly, a peculiar drop in solution consumption of about 20-30% and an increase in food intake of about 30% were observed in all groups during the last few days before offspring delivery (data not shown).

Litter characteristics are summarized in Table 2. Prenatal nicotine exposure had a strong teratogenic effect on virtually all measures used in the present study, except the number of live litters and offspring sex ratio. Between-group differences in litter size ($F(3,41)=11.10$, $p<0.001$), and the numbers of females ($F(3,41)=4.18$, $p<0.02$) and males ($F(3,41)=7.00$, $p<0.001$) per litter, were a result of decreased numbers of offspring in the 0.06 and 0.08 mg/ml groups as compared with control animals; litter size also decreased in the 0.04 mg/ml group. Most of the litters prenatally exposed to nicotine were killed either by their biological or foster mothers. There was no difference in this parameter between the groups prenatally exposed to nicotine. In contrast, no animals were killed in control litters. In consequence, both the percent of affected litters (malformed, dead or killed pups) per group ($F(3,41)=18.07$, $p<0.001$) and percent of affected pups per litter ($F(3,41)=8.32$, $p<0.001$) were significantly higher in groups prenatally exposed to nicotine comparing to control animals. There was no sex difference in the number of affected pups per litter.

Postnatal growth and maturation—Body weight gain is shown in fig. 1 A. A significant main effect of prenatal nicotine exposure on body weight ($F(3,19)=7.34$, $p<0.01$) was dependent on postnatal day (group x PND interaction, $F(12,196)=3.28$, $p<0.001$). Post-hoc analysis revealed that all groups exposed to nicotine had lower body weight at birth and

during the first five PND. In addition, the 0.08 group displayed lower body weight on PND 5, 10 and 15 in comparison to all other groups, and on PND 20 comparing to Con and 0.04 groups. All other maturational measures were delayed by prenatal exposure to nicotine (Figure 1). Significant main effects of group for pinnae detachment ($F(3,19)=27.2$, $p<0.001$) and fur appearance ($F(3,19)=65.4$, $p<0.001$) resulted from delayed maturation in the 0.08 group comparing to all other groups of animals (Fig. 1B and 1C). Incisor eruption (Fig. 1D) was significantly delayed in all nicotine exposed groups ($F(3,19)=12.2$, $p<0.001$). Delayed eye opening ($F(3,19)=11.9$, $p<0.001$; Fig. 1E) was observed in 0.08 and 0.04 groups. There was no effect of sex or significant interaction between sex and group or PND in any of the maturational measures.

Neurobehavioral development—The ontogeny of righting reflex was delayed in rats prenatally exposed to nicotine (fig. 2A). There were significant main effects of PND ($F(3,153)=54.6$, $p<0.001$) and group ($F(3,19)=33.1$, $p<0.001$), and PND x group interaction ($F(9,153)=6.2$, $p<0.001$). Rats in all groups showed decreased latencies to right themselves onto all four feet from a supine position over the consecutive sessions. *Post-hoc* analyses revealed that latencies to turn were longer in group 0.08 mg/ml in comparison to all other groups, and in the 0.06 mg/ml group as compared with Con animals and the 0.04 mg/ml group. There was no effect of sex or significant interaction between sex and group or PND in this measure. There was also no difference between the 0.04 mg/ml and the control group.

The ontogeny of negative geotaxis was significantly delayed in rats prenatally exposed to nicotine (fig. 2B). There were significant overall main effects of PND ($F(3,153)=28.3$, $p<0.001$) and nicotine exposure ($F(3,19)=22.8$, $p<0.001$), but not for the PND x group interaction. All groups decreased the latencies to turn 180° over the consecutive sessions. *Post-hoc* analyses revealed that latencies to turn were longer in all groups exposed to nicotine in comparison to control animals, and in 0.08 mg/ml group in relation to groups 0.04 and 0.06. There was no effect of sex or significant interaction between sex and group or PND in this measure. There was also no difference between the 0.04 mg/ml and control group.

There was an overall significant main effect of group on grip strength ($F(3,19)=17.7$, $p<0.001$; fig. 2C). *Post hoc* analyses revealed decreased strength in groups 0.08 and 0.06 mg/ml comparing to Con animals, and in group 0.08 mg/ml in comparison with the 0.04 mg/ml group. There was no effect of sex or significant interaction between sex and group or PND in this measure. There was also no difference between the 0.04 mg/ml and the control group.

For locomotor activity on PND19 (fig. 2D) there was no difference between groups or genders, and no significant interaction, for numbers of cage crosses during the 30 minute session.

Discussion

In preclinical studies an empirical determination of the optimal dosage to elicit species-specific responses is essential. In the pilot experiment, the lowest and medium concentrations of nicotine (0.03 and 0.06 mg/ml) led to daily nicotine consumption of 2.7 and 5.4 mg/kg with mean nicotine plasma levels of 10.7 to 60 ng/ml. These results correspond with human data showing that peak plasma nicotine concentrations, for typical smokers, are in the range 10-50 ng/ml (Benowitz and Jacob, 1997). Our results also agree with other studies using oral nicotine exposure in pregnant rodents (Peters and Tang, 1982; Maehler et al., 2000; Paz et al., 2007), in which nicotine doses of 2–6 mg/kg/day resulted in nicotine plasma levels in the range of moderate to heavy smokers (Benowitz et al., 1990).

As reported by Murrin et al. (1987) higher concentrations of nicotine solution (0.1 and 0.12 mg/ml) led to large drops in fluid consumption and decreases in weight, strongly suggesting that these concentrations should not be used for prolonged periods, especially that we found no difference in nicotine blood levels between groups exposed to doses of 0.6 and 0.1 mg/ml, which can be explained by the drop in solution consumption at the higher concentration of nicotine.

During the main experiment, four weeks of pre-exposure to increasing doses of nicotine as the only source of water resulted in decreased body weight gain and lower solution consumption in all groups exposed to nicotine. Dose-dependent nicotine ingestion was observed for the final concentrations of nicotine solution. During pregnancy, solution consumption was lower in groups exposed to nicotine, but body weight gain was compromised only at the higher doses (0.06 and 0.08 mg/ml). Dose-dependent nicotine ingestion was observed with mean daily consumption ranging from 2.9 to 4.4 mg/kg. In line with previous studies (Murrin et al., 1987) the effects of nicotine on food consumption were not as dramatic as the effects on water consumption, but they were statistically significant with the 0.08 mg/ml group eating less during pregnancy in comparison to all other groups. The difference in body weight gain during pregnancy might have been partially mediated by the smaller number of animals born by females exposed to nicotine and the lower body weight of offspring.

Litter characteristics

Prenatal exposure to nicotine had no effect on the number of live litters but significantly and dose-dependently decreased litter size, with the 0.06 and 0.08 mg/ml groups both affected. The sex ratio was not affected. These results support previous studies (Romero and Chen, 2004; Farkas et al., 2006). In contrast, the dramatic effects of prenatal exposure to nicotine on the number of offspring being malformed, dead, killed or neglected by biological and foster mothers have not been reported. Previous studies using similar doses of nicotine (3-6 mg/kg/day) delivered in tap water (Peters and Ngan, 1982; Peters and Tang, 1982) showed no impact of oral nicotine ingestion on litter size. Strain differences (Lister hooded in our study vs. Sprague Dawley and Fisher 344 in cited literature) and the prolonged time of pre-exposure to nicotine solution before mating (6 weeks vs. 4 weeks in our study) may account for some of these discrepancies. The method of animal assignment to particular nicotine solutions used in the present study, based on their actual consumption/tolerance to nicotine effects, might be another reason. Potential interaction between tolerance to nicotine effects in dams and teratogenic effects of nicotine exposure in offspring should be explored further.

Maturation of offspring

Characteristics of nicotine teratogenicity include lower birth weight and decreased weight gain. Birth weight represents an accurate index of prenatal development. Similar to the results of human studies (Eskenazi et al., 1995), findings in animals consistently show lower birth weights in offspring exposed to nicotine *in utero* and slower postnatal weight gain (Leichter, 1995; Paulson et al., 1993). In our study, birth weight was decreased in all groups prenatally exposed to nicotine. Body weight gain was compromised in the 0.08 group during the first 20 PNDs, and in the 0.04 and 0.06 mg/ml groups during the first 5 PNDs. Interestingly, prenatally nicotine-exposed children also recovered their body weights over time (Day et al., 1994). The long-term functional significance of lower birth weight is still unclear, but studies in humans tend to show associations between low birth weight and long-term cognitive deficits (Breslau et al., 1994; Hack et al., 2006; Gianni et al., 2007). This aspect will be investigated in our future studies. All other developmental measures used in the present experiment were compromised in offspring prenatally exposed to nicotine; however, groups 0.04 and 0.06 were spared on most measures except a delay in eye lid

opening and incisor eruption, respectively. The most severely affected 0.08 mg/ml group displayed delay in pinnae detachment, fur appearance, incisor eruption and eye lid opening. Our results are in accordance with Ajarem and Ahmad (1998), who showed reduced postnatal body weight gain, significant delay in eye opening and the appearance of body hair in mice prenatally exposed to nicotine (daily s.c. injections of 0.05 mg/kg).

Development of offspring

Regular observation of reflex ontogeny is a sensitive indicator of adaptation of the neonate in the early developmental stages. In our study, physical retardation and neuronal incapacity for motor control in rats prenatally exposed to nicotine were expressed as a significant delay of the righting reflex and negative geotaxis, as well as a shorter latency to fall in grip strength test in group 0.06 and 0.08 mg/ml, suggesting impairment of motor coordination and muscle strength. Our results are in line with previous studies showing deficits in righting reflex and negative geotaxis in rats and mice at similar doses (Peters and Ngan, 1982; Ajarem and Ahmad, 1998). The delay in attaining these skills is probably due to damage or poor development of the motor and vestibular systems of the brain, and decreased somatic growth. Locomotor activity assessed on PND19 was not affected in offspring prenatally exposed to nicotine. This is in contrast to some previous studies (Tizabi et al., 1997; Pauly et al., 2004), but not others (Martin and Becker, 1970; LeSage et al., 2006), and might be related to different doses of nicotine, to routes of administration (osmotic minipumps vs. oral ingestion), or age of testing (PND19 as compared with PND57). Better understanding of this phenomenon requires further studies.

Study Limitations

The present study suffered from a number of limitations. Firstly, the possible teratogenic effects of prenatal exposure to nicotine cannot be conclusively distinguished from potential effects of dehydration and any associated vasoconstriction and hypoxia. Water restriction during pregnancy induced marked alterations in maternal-fetal fluid homeostasis and resulted in low birth weight in newborns (Ross and Desai, 2005). Although it was suggested that these compensatory changes contributed to the maintenance of pregnancy and finally led to an increased adaptive response to future dehydration in offspring, their interaction with nicotine effects cannot be excluded, especially the potential stressful effect of prolonged water restriction. However, a significant degree of dehydration is also observed in pregnant rats given ethanol as the sole source of fluid prior to and during gestation, and it has been shown that in this case dehydration does not contribute significantly to retarded fetal growth in the offspring (Leichter and Lee, 1984). Direct tests on the behavioural effects of gestational dehydration in rats do not seem to have been published and therefore this issue will be addressed in future work using a pair-feeding design. Secondly, the method for assigning animals to particular nicotine solutions, based on their actual consumption of and tolerance to nicotine's effects, might influence and to some extent confound the dose-related nature of the teratogenic effects. The way animals were divided into groups was a major difference between our study and previous reports on gestational exposure to nicotine solutions (Peters and Ngan, 1982; Peters and Tang, 1982; Paz et al., 2007) and showing only minimal teratogenic effects in offspring. The potential interaction between tolerance to nicotine's effects in dams and teratogenic effects should be explored further. Thirdly, the small numbers of litters and animals in the 0.08 mg/ml group restricts the conclusions; however, strong teratogenic effects of this concentration and its unsuitability for gestational studies have been clearly shown.

Conclusions

Maternal cigarette smoking during pregnancy occurs in up to one-fourth of all pregnancies in the world (Mckay and Eriksen, 2006), contributes significantly to adverse developmental outcomes in children (reviewed in Slotkin, 2007). Animal studies have provided compelling evidence that nicotine is the single most important factor triggering the negative effect on neurodevelopment (Slotkin, 2007; Ernst et al., 2001). The present study found that pregnant females will ingest nicotine in drinking water at levels that provide nicotine intake and nicotine blood levels comparable to the ones reported in moderate and heavy smokers. We have shown that rats will well tolerate nicotine solution at concentrations below 0.04 mg/ml with some moderate adverse effects at 0.06 mg/ml. Concentrations above 0.08 mg/ml are not recommended since they lead to very large drops in fluid consumption and body weight. The strong teratogenic effects of prenatal nicotine exposure described in the present study suggest that concentrations above 0.06 mg/ml should be avoided in gestational studies. Teratogenic effects of prenatal nicotine exposure on mortality, maturation and development of the pups show that oral exposure might have more deleterious effects than minipumps, and necessitates further studies to better understand this phenomenon. Nevertheless, this model provides an approach for examining teratogenic risk associated with prenatal nicotine exposure and can be utilised for investigating the neuroanatomical and biochemical alterations underlying associated functional deficits.

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Figure 1.

Decreased body weight (A) and delayed maturation (B-D) in offspring whose mothers were exposed to different concentrations of nicotine in tap water as the only source of fluid for four weeks before mating and during pregnancy. Data are shown as means + SEM. Litter (Con=12, 0.04=5, 0.06=5, 0.08=4) was used as the unit for analyses. *Post hoc* Least Significance Difference Test results significant at least at $p < 0.05$ are marked as follows: * vs. Con, + vs. 0.04 mg/ml, § vs. 0.06 mg/ml group.

Figure 2.

Impairment of motor coordination (A, B) and muscle strength (C) in offspring whose mothers were exposed to different concentrations of nicotine in tap water as the only source of fluid for four weeks before mating and during pregnancy. There was no impact of prenatal nicotine exposure on the offspring's locomotor activity on PND 19 (D). Data are shown as means + SEM. Litter (Con=12, 0.04=5, 0.06=5, 0.08=4) was used as the unit for analyses. *Post hoc* Least Significance Difference Test results significant at least at $p < 0.05$ are marked as follows: * vs. Con, + vs. 0.04 mg/ml, § vs. 0.06 mg/ml group.

Table 1

Characteristics of females exposed to nicotine solution in tap water as the only source of fluid for four weeks before mating and during pregnancy. Data (means $\pm$ SEM) are shown for the last week before mating and the whole pregnancy, when the final concentrations of nicotine were used

Final nicotine concentration	0.00 mg/ml (n=15)	0.04 mg/ml (n=16)	0.06 mg/ml (n=14)	0.08 mg/ml (n=16)	p
Number of pregnant females @	15	14	14	14	ns
Body weight before exposure	176.4 $\pm$ 4.0	179.7 $\pm$ 3.2	177.0 $\pm$ 3.8	170.4 $\pm$ 2.6	ns
Percent weight gain during habituation	20.8 $\pm$ 1.4	7.1 $\pm$ 1.1 *	4.7 $\pm$ 1.1 *	4.8 $\pm$ 1.0 *	<0.001
Maternal weight on GD1	214.4 $\pm$ 4.4	195.1 $\pm$ 3.47 *	187.2 $\pm$ 3.4 *	182.1 $\pm$ 3.1 *	<0.001
Percent weight gain during pregnancy	40.5 $\pm$ 3.5	37.8 $\pm$ 3.2	26.1 $\pm$ 3.8 *	17.7 $\pm$ 3.8 * ⁺	<0.001
Nicotine intake during habituation (mg/kg/day) in last week of pre-exposure	n/a	3.6 $\pm$ 0.1	5.2 $\pm$ 0.12 ⁺	6.2 $\pm$ 0.2 ⁺ [§]	<0.001
Nicotine intake during pregnancy (mg/kg/day)	n/a	2.9 $\pm$ 0.1	3.7 $\pm$ 0.1 ⁺	4.4 $\pm$ 0.2 ⁺ [§]	<0.001
Food intake during habituation (g/kg/day) in last week of pre-exposure	63.6 $\pm$ 1.1	54.3 $\pm$ 4.2	59.7 $\pm$ 1.8	54.2 $\pm$ 2.0	<0.03
Food intake during pregnancy (g/kg/day)	86.6 $\pm$ 1.5	85.2 $\pm$ 1.9	86.0 $\pm$ 2.5	76.6 $\pm$ 2.8 [§]	<0.02
Solution intake during habituation (ml/kg/day) in last week of pre-exposure	116.3 $\pm$ 3.4	90.8 $\pm$ 3.2 *	86.2 $\pm$ 2.0 *	77.9 $\pm$ 2.4 * ⁺	<0.001
Solution intake during pregnancy (ml/kg/day)	143.3 $\pm$ 2.8	70.3 $\pm$ 2.8 *	61.7 $\pm$ 2.1 *	54.9 $\pm$ 1.9 * ⁺	<0.001

Note: One dam from the NIC 0.04 mg/ml group died from reasons not related to the experiment and is not included in the analysis. All p values are from the overall analysis. Post hoc test results significant at least at 0.05 are marked as follows:

* vs. Con

⁺ vs. 0.04

spermatozoa found after mating

@

vs. all groups

\$

90.00%

\$

Table 2
Characteristics of litters exposed to different doses of nicotine during gestation. Data are shown as mean $\pm$ SEM

Nicotine dose	NIC 0.00 mg/ml	NIC 0.04 mg/ml	NIC 0.06 mg/ml	NIC 0.08 mg/ml	<i>p</i>
Number of live litters	12	11	11	11	ns
Litter size	11.33 $\pm$ 0.57	9.55 $\pm$ 0.87	6.45 $\pm$ 0.79 ^{*/+}	6.00 $\pm$ 0.85 ^{*/+}	<0.001
Number females	5.58 $\pm$ 0.66	4.73 $\pm$ 0.52	3.27 $\pm$ 0.62 [*]	3.40 $\pm$ 0.48 [*]	<0.02
Number males	5.75 $\pm$ 0.37	4.82 $\pm$ 0.64	3.18 $\pm$ 0.58 [*]	2.91 $\pm$ 0.44 [*]	<0.001
Sex ratio (% males)	52.1 $\pm$ 4.1	49.3 $\pm$ 4.4	50.3 $\pm$ 7.7	52.7 $\pm$ 5.5	ns
Litters killed by biological mothers	0	3	2	4	---
Litters killed or rejected by foster mothers	0	3	4	3	---
Percent affected litters (malformed, dead or killed pups)	0	54.5 [*]	63.6 [*]	100 ^{*/+}	<0.001
Percent affected pups per litter (malformed, dead or killed pups)	0	37.2 $\pm$ 14.1 [*]	29.8 $\pm$ 9.7 [*]	67.4 $\pm$ 11.1 ^{*/+} &	<0.001
Number of affected males/females per group (malformed, dead or killed)	0	29/24	22/18	27/27	---

All *p* values are from the overall analysis. *Post hoc* test results significant at least at 0.05 are marked as follows:

^{*} vs. Con

⁺ vs. 0.04 group