Hepatotoxicity of Prenatal and Postnatal Exposure to Nicotine in Rat Pups

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Prenatal and postnatal exposure to nicotine have been shown to affect developing tissues in growing animals. Rat pups were exposed to nicotine prenatally and/or postnatally for 10 days by feeding pregnant and lactating rat dams water containing 0, 54, or 108 μM of nicotine. Nicotine exposure did not affect either litter sizes or body weights at birth and at 10 days of age. Exposure to 108 μM of nicotine prenatally increased significantly the incidence of focal necrosis at birth, and the liver damage was still evident at 10 days of age even after the pups were allowed to suckle dams not exposed to any nicotine during the study period. Continuation of nicotine exposure postnatally increased the incidence and severity of focal and confluent necrosis. Postnatal exposure to 108 μM of nicotine to pups not previously exposed also increased the incidence of mild focal and confluent necrosis, although not significantly. Exposure to nicotine prenatally did not affect liver malondialdehyde (MDA) levels at birth. However, liver MDA was significantly lower in rat pups exposed to nicotine prenatally when they were 10 days of age irrespective of whether there were further exposure to nicotine postnatally. Reasons for the late onset of the low MDA levels need further investigation. Postnatal nicotine exposure to either 54 or 108 μM of nicotine to pups not previously exposed fails to affect liver MDA at 10 days of age. The significant decrease in hepatic superoxide dismutase (SOD) levels reflects those of hepatic injury, indicating the possibility of a nicotine-induced downregulation of SOD enzyme production.

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Prenatal and postnatal exposure to cigarette smoke and nicotine have been shown to affect pregnancy outcome (1, 2) and to have detrimental effects on the development of fetuses and neonates (3, 4). Nicotine exposure is also associated with a variety of organ damage (5–7). The effects of nicotine include modulation of enzyme activities in the lung, kidney, and liver (5, 8).

There is increasing evidence that cellular damage that occurs with nicotine exposure is associated with an imbalance in the cellular oxidant-antioxidant system (9–11). Nicotine administration induces ischemia in gastric mucosal damage by the production of superoxide free radicals (12) and the pathogenesis of atherosclerosis in rats by increased lipid peroxidation (9). Lipid peroxidation is also a cause of oxidative stress when pancreatic tissue or esophageal mucosa is incubated with nicotine (10, 11). Defenses against these oxygen free radicals are several antioxidant enzymes, including superoxide dismutase (SOD). SOD, an oxygen free radical scavenging enzyme, has been shown to be a cellular protective enzyme in bowel ischemia and gastric lesion formation (13, 14), and in studies using pancreatic tissues or esophageal mucosa incubated with nicotine (10, 11).

Previous studies have indicated that nicotine is potentially hepatotoxic, as shown by the histomorphological changes in the liver in nulliparous female rats and in pregnant rats (15). However, no data are available on the hepatotoxic effects of nicotine on the fetus or newborn when nicotine is administered to the mothers during pregnancy or lactation. We expect nicotine to have an effect on the offspring, as it has been shown that prenatal and postnatal exposure to cigarette smoke in rats increases the levels of microsomal aryl hydroxycarbon hydroxylase in the perinatal livers (8). Therefore, we postulate that prenatal and postnatal exposure to nicotine increases the incidence of hepatotoxicity in the fetus and neonates; that nicotine exposure reduces liver SOD level, thus decreasing the ability of the liver to handle oxidative stress; and that nicotine exposure increases oxidative stress as manifested by an increase in lipid peroxidation, that is, increase in malondialdehyde (MDA) level. Our objectives, therefore, are to evaluate, from changes in liver histomorphology, the hepatotoxicity in rat pups exposed to nicotine via placental transfer and/or via transfer from milk, and to identify the role of SOD and MDA in the production of liver damage.

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Materials and Methods

Experimental Design. Twenty-seven 3-month-old, nongravid, female Sprague-Dawley rats (Laboratory Animal Unit, The University of Hong Kong) were housed individually in polycarbonate cages in a room on a 12:12-hr dark:light cycle and the temperature was controlled at 22° ± 1°C. They had free access to standard rat chow (PMI Feeds, Inc., St. Louis, MO) and to water containing one of three doses of dissolved nicotine hydrogen tartrate (Sigma, St. Louis, MO) 10 days prior to their timed conception and through to 10 days postpartum. The concentrations of nicotine salt in tap water were 0, 54, and 108 μM for groups C, LN, and HN, respectively. Nine rats were randomly assigned to each group. Addition of nicotine salts to drinking water will reduce significantly consumption of these fluids by 14% for those drinking the 54 μM solution and 28% for those drinking the108 μM solution, resulting in their respective nicotine intake of 2.6 ± 0.1 mg/kg/day and 4.3 ± 0.3 mg/kg/day (16). Body weights were recorded three times weekly. On the day of birth, litter sizes and birth weights of pups were recorded. Nine pups from each litter were kept for another 10 days for further study and the remaining pups were studied for their liver damage at birth.

To study the effects of prenatal exposure to nicotine only, both prenatal and postnatal exposure to nicotine, or postnatal exposure to nicotine of pups not previously exposed to nicotine prenatally, the nine surviving pups from each litter were randomly regrouped into three groups such that three pups from each litter were reassigned to lactating dams of the three different groups. In this way, each lactating dam had three pups from her own litter and three pups from each of the other two litters to nurse. Identification of pups was achieved by marking their tails. Body weights of the lactating dams and pups were recorded three times weekly. At 10 days of age, the pups were studied for liver damage. The protocol was approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong.

Study of Liver Damage. On the day of study, rat pups were anesthetized with pentobarbital sodium i.p. (30 mg/kg body wt). The liver was removed rapidly, and a portion was dissected and frozen in liquid nitrogen and was then stored in a freezer at −70°C until analysis of SOD and MDA. An aliquot from another portion of the liver from only one pup from each litter was preserved in 10% buffered formaldehyde for histologic examination of liver damage. Only pups in group C and group HN were evaluated histologically for liver damage.

Histological evaluation. After tissue fixation, the liver samples were embedded in paraffin wax and four microsections were prepared and stained with hematoxylin and eosin solution. The samples were coded to avoid bias and were evaluated and graded. Two parameters, focal necrosis and confluent necrosis, were selected as indicators of liver injury (15). Focal necrosis was considered to be present in the liver sample when individual or small groups of cells were shown to undergo necrosis. The cytoplasm of cells undergoing necrosis became intensely eosinophilic and the nucleus underwent pyknotic changes or fragmentations, resulting in the formation of acidophil bodies. These foci were usually accompanied by an infiltration of intense inflammation. Confluent necrosis was present when either large groups of cells or a confluent area of cells underwent necrosis. To quantify the extensiveness of the morphological changes, liver sections were graded for each of the criteria using an arbitrary scale of 0, 1, 2, and 3, for no injury, mild, moderate, and severe, respectively (15).

Measurement of SOD activity. Hepatic SOD was determined by spectrophotometry (17). The liver sample was homogenized in a ratio of one part of tissue to nine parts of 0.05 M potassium phosphate buffer, and was centrifuged at 27,000*g* for 20 min to remove particulate matter. The SOD activity was determined using the nitroblue tetrazolium (NBT) reaction. The reaction was stopped by the addition of $CuCl_2$ solution. Inhibition of NBT reduction was determined spectrophotometrically at 560 nm. Activity of SOD was expressed as per unit weight of protein determined by the Lowry method (18).

Measurement of MDA levels. Hepatic MDA level was determined by the method of Ohkawa *et al.* (19). Briefly, an aliquot of the liver samples from each pup was homogenized in 1.15 M KCl solution for 30 sec. To 0.1 ml of the homogenized liver, 200 μl of 8.1% thiobarbituric acid and 0.7 ml of distilled water were added. The resultant mixture was boiled at 95°C for 1 hr. After cooling, the mixture was centrifuged at 2450*g* for 10 min. The absorbance of the red supernatant was measured at 532 nm using a reagent blank, and the MDA concentrations were calculated from a standard curve with known tetramethoxypropane concentrations. Concentration of MDA was expressed as per unit weight of protein determined by the Lowry method (18).

Data Analysis. Except for histological evaluation, data from pups within each litter at birth were pooled and averaged, and the mean was considered as $n = 1$. For analysis of data at 10 days of age, data from the three pups per litter from each prenatal exposure group were pooled and averaged, and the mean was considered as $n = 1$. Therefore, in all statistical analysis, n equals the number of litters from each prenatal exposure group. One-way analysis of variance (ANOVA) was used to detect differences among groups. When significant differences were detected, a pairwise comparison analysis using Student-Newman-Keuls test was done. Data were presented as mean ± SEM. To test for the effects of nicotine on changes in liver histomorphology, data on mild, moderate, and severe histological damages were grouped together for analysis of statistical differences with those from group C (0 μM) using the Fisher exact test. All analyses were performed with SigmaStat statistical software (SigmaStat, Version 1.0, Jandel Scientific,

San Rafael, CA). All differences were considered significant when $P < 0.05$.

Results

There were no differences in the mean maternal body weights among groups throughout the whole study period. Body weights for the three groups of female rats ranged from 264 ± 3 g to 275 ± 5 g at the start of the study, 429 ± 13 g to 435 ± 10 g immediately after birth, and 335 ± 15 g to 341 ± 11 g at 10 days postpartum. There were also no differences among groups in their weight gain during the 20 days of pregnancy (ranging from 156 ± 16 g to 160 ± 12 g). Similarly, no differences in mean pup weights were detected among groups at birth and at 10 days of age. Body weights for the three groups of pups ranged from 7.0 ± 0.1 g to 7.2 ± 0.1 g at birth and 24.8 ± 0.9 g to 26.8 ± 1.4 g at 10 days of age. There were also no differences in the mean litter sizes among groups (14.6 ± 0.6 to 15.0 ± 0.6 pups/litter).

Histological changes in the liver of pups showed that prenatal exposure to 108 μM of nicotine (group HN) increased significantly the incidence of focal necrosis, but not confluent necrosis, at birth (Table I). Focal necrosis incurred *in utero* was still evident at 10 days of age even after the pups were allowed to suckle dams not exposed to any nicotine (group HN, 0 μM) during the study period (Table II). However, continuation of exposure of pups in group HN to nicotine by letting them suckle dams drinking 108 μM nicotine (group HN, 108 μM) increased significantly the severity and incidence of focal necrosis (Table II and Fig. 1A). Incidence and severity of confluent necrosis increased also, but not significantly (Table II and Fig. 1B). Postnatal exposure to 108 μM of nicotine to pups not previously exposed to nicotine (group C, 108 μM) also increased the incidence of mild focal and confluent necrosis, although the increase was not significant.

Prenatal exposure to nicotine (groups LN and HN) decreased liver SOD levels at birth, although only those in group LN were significant ($P < 0.05$), and those in group HN were not ($P = 0.07$; Table I). After 10 days of nonexposure to nicotine, there were no differences in liver SOD levels among groups, irrespective of whether there were previous exposure to nicotine *in utero* (Table III). Postnatal exposure of 54 μM did not affect liver SOD levels for all three groups. However, postnatal exposure to the higher nicotine levels of 108 μM caused a significant decrease ($P < 0.05$) in liver SOD levels in group HN when compared with that of group C (0 μM). Hepatic SOD levels were reduced, but not significantly ($P = 0.06$), in group C (108 μM) and group LN (108 μM) when compared with that of group C (0 μM).

There were no differences in hepatic MDA levels among groups at birth (Table I). However at 10 days of age, pups in groups LN (0 μM) and HN (0 μM), which were not exposed to any nicotine after birth, had lower hepatic MDA levels than those of group C (0 μM) at 10 days of age, but only those of group HN (0 μM) were significantly different (Table III). Continuation of nicotine exposure of either 54 or 108 μM did not change the MDA levels in group HN, but did further decrease the MDA levels of pups in group LN so that the levels were significantly lower than those in group C (0 μM).

Discussion

Our study shows that litter sizes, litter weights at birth, and weight gain after birth were not affected by maternal exposure to nicotine during the pregnancy and lactation period. These results contrasted with those of Shacka and coworkers in rats (1) and those in infants born to smokers (2). Both of these studies have shown poor pregnancy outcome and decreased weight gain after birth. One possible explanation for our differences with the human study is that the nutritional status of our rat dams was not affected, as there were no differences among groups in their pre-pregnancy weights, weight gain during pregnancy, and weight changes during lactation. In humans, smokers have significantly lower pre-pregnancy weight (2, 20) and lower body mass index (2) compared with nonsmokers. Pre-pregnancy weight, body mass index, and pregnancy weight gain all independently affect birth weight (2, 20). Even after

Table I. Histological Changes, Hepatic Superoxide Dismutase (SOD), and Malondialdehyde (MDA) Levels of Rat Pups at Birth

Groups	Prenatal nicotine exposure	Histological changes[a]				SOD (U/mg protein)	MDA (nmol/mg protein)
		FN		CN			
		0	1 2	0	1 2		
GpC	0 μM	8[b]	1 0	9	0 0	18.5 ± 1.3	11.1 ± 0.9
Gp LN	54 μM	—	— —	—	— —	15.1 ± 0.9[c]	10.9 ± 0.8
Gp HN	108 μM	4	5 0‡	9	0 0	15.9 ± 0.6	11.1 ± 0.8

Note. Gp C, Gp LN, and Gp HN denote dams drinking the respective nicotine solution, 0, 54, and 108 μM. Data for SOD and MDA are means ± SEM; $n = 9$ litters/exposure group (see data analysis for description).

[a] Histological changes: FN, focal necrosis; CN, confluent necrosis. Severity of liver damage: 0, nil; 1, mild; 2, moderate (see Materials and Methods for description).

[b] Number of pups exhibiting pathology of the liver. Pups with liver damage were grouped together for analysis of differences with those of Group C (see "Data analysis" for description).

[c] Significantly different from Group C; $P < 0.05$.

Table II. Histological Changes in Livers of Rat Pups Exposed to Nicotine Prenatally and/or Postnatally

Groups	Prenatal nicotine exposure	Postnatal exposure for 10 days							
		0 μM				108 μM			
		FN[a]		CN		FN		CN	
		0	I1 2I	0	I1 2I	0	I1 2I	0	I1 2I
Gp C	0 μM	9[b]	I0 0I	9	I0 0I	5	I3 0I	6	I2 0I
Gp HN	108 μM	3	I6 0I[c]	8	I1 0I	0	I6 3I[c]	6	I2 1I

Note. Gp C, Gp LN, and Gp HN denote dams drinking the respective nicotine solution, 0, 54, and 108 μM.
[a] FN, focal necrosis; CN, confluent necrosis. Severity of liver damage: 0, nil; 1, mild; 2, moderate (see "Materials and Methods" for description).
[b] Number of pups exhibiting pathology of the liver. Pups with liver damage were grouped together for analysis of differences with those of Group C (see "Data analysis" for description).
[c] Significantly different from Group C (0 μM); $P < 0.05$.

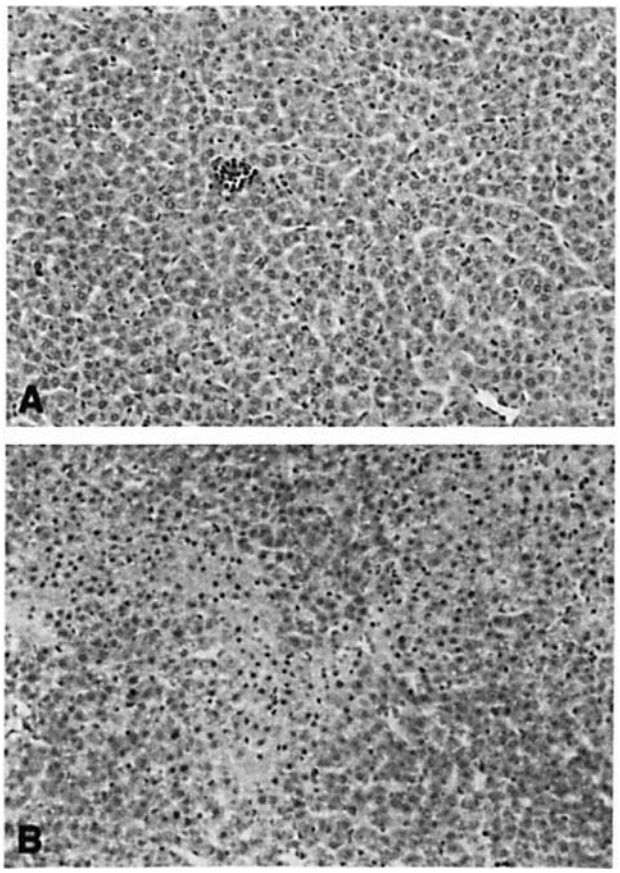

Figure 1. (A) Grade I focal necrosis in 10-day-old rat pups after exposure to nicotine throughout the gestation period and suckling dams drinking 108 μM nicotine solution. Hematoxylin and eosin stain. (B) Grade II confluent necrosis in 10-day-old rat pups after exposure to nicotine throughout the gestation period and suckling dams drinking 108 μM nicotine solution. Hematoxylin and eosin stain.

adjusting for maternal nutritional status, the negative influence of smoking on birth weight still exists (2).

We cannot explain the differences between our findings with those of chronic smokers and rats in Shacka's studies (1), as the differences cannot be attributed to lower plasma levels of nicotine attained by our rat dams and a lower exposure of fetuses to nicotine. Although plasma nicotine levels were not measured in the present study, previous studies in our laboratory have shown that plasma nicotine levels of adult female rats were 27.1 ± 1.3 ng/ml for those drinking the 54 μM nicotine solution, and 36.1 ± 3.7 ng/ml for those drinking the 108 μM nicotine solution (D. Wong and C-H. Cho). Plasma nicotine levels achieved by our rats drinking the higher nicotine solution are comparable to the level of 38 ng/ml as reported by Shacka and coworkers (1), and are equivalent to levels found in chronic smokers who smoke two packs of cigarettes a day (1).

Although birth weights and litter sizes are not affected by prenatal exposure to nicotine, our study shows that maternal exposure to 108 μM of nicotine causes a significant increase in the incidence of mild focal necrosis in the pups at birth. This indicates injury of some liver cells at birth. Incidence of focal necrosis persists after postnatal withdrawal from nicotine exposure for 10 days. Postnatal nicotine exposure for 10 days to pups not exposed prenatally causes a slight increase, although not significantly, in the incidence of mild liver damage, as observed by the occurrence of mild focal necrosis and mild confluent necrosis. It is of interest that the extent of damage in our pups caused by either prenatal or postnatal exposure to nicotine was less severe than those in nonpregnant and pregnant rats who drank a solution containing 108 μM of nicotine for 10 days (15). The less severe liver damage can be explained by a lower plasma concentration of nicotine in the rat pups compared with the adults. Although it is well established that fetuses acquire nicotine via placental transfer, the amount of fetal exposure to nicotine is expected to be less than that of their mothers. Fetal acquisition and accumulation of nicotine have been studied by analysis of hair samples from newborn infants of mothers who smoke during the prenatal period (21). These investigators have found a lower nicotine level (0.15–11.80 ng/mg) in hair samples from the neonates when compared with that of their mothers (0.37–63.50 ng/mg), indicating a lower exposure or accumulation of nicotine by the fetus. Similarly, although nicotine is secreted into breast milk, we expect the exposure of the rat pups to

Table III. Hepatic SOD and MDA Levels of Rat Pups at 10 Days of Age

Groups	Prenatal nicotine exposure	SOD (U/mg protein)			MDA (nmol/mg protein)		
		Postnatal nicotine exposure for 10 days					
		0 μM	54 μM	108 μM	0 μM	54 μM	108 μM
Gp C	0 μM	24.6 ± 1.4	25.6 ± 1.4	19.7 ± 1.8	9.2 ± 0.6	9.4 ± 0.7	9.7 ± 0.9
Gp LN	54 μM	23.2 ± 1.0	25.4 ± 1.3	19.8 ± 1.8	7.8 ± 0.7	7.3 ± 0.7[b]	7.4 ± 0.6[b]
Gp HN	108 μM	22.5 ± 1.1	20.3 ± 1.3[a]	19.2 ± 2.1[a]	7.0 ± 0.7[b]	7.4 ± 0.6[b]	7.5 ± 0.5[b]

Note. Gp C, Gp LN, and Gp HN denote dams drinking the respective nicotine solution, 0, 54, and 108 μM. Data are means ± SEM; $n = 9$ litters/exposure group (see "Data analysis" for description).
[a] Significantly different from Gp C (0 μM) for SOD; $P < 0.05$.
[b] Significantly different from Gp C (0 μM) for MDA; $P < 0.05$.

nicotine to be much less than that of their mothers, as the oral bioavailability of nicotine has been reported to be less than 20% (22). As expected, our study shows that the highest incidence of liver damage, as shown by the marked increase in the incidence and severity of focal necrosis, occurred in pups exposed to nicotine both prenatally and postnatally. Studies in adult female rats have shown that even though moderate degrees of focal necrosis and confluent necrosis were present after 10 days of exposure to 108 μM nicotine, no significant changes in their serum glutamic-pyruvic transaminase (SGPT) can be detected (15). Therefore, it is reasonable to assume that the functions of the liver in our rat pups are not compromised greatly, even though a certain degree of hepatic injury has occurred.

Contrary to our expectation, hepatic injury in our rat pups was not associated with a parallel increase in hepatic MDA levels, an indicator of lipid peroxidation. Increase in lipid peroxidation has been reported in nicotine-administered rats (9) and in pancreatic tissue (10), esophageal mucosa (11), and Chinese hamster ovary cells (23) when incubated with nicotine. In our study, prenatal exposure to either the lower or higher dose of nicotine did not affect the hepatic MDA levels of pups, although a significant increase in incidence of mild focal necrosis occurred in pups exposed to the higher level of nicotine. This result is similar to those of Gogo and coworkers (16), who reported no change in hepatic MDA levels in fetuses of dams drinking either 54 or 108 μM of nicotine for 10 days. These investigators also reported no change in hepatic MDA levels in their mothers (16), although those drinking the higher concentration of nicotine showed occurrence of moderate focal necrosis and mild confluent necrosis (15).

That hepatic MDA levels do not reflect mild focal and confluent necrosis is also seen in the 10-day-old pups. At 10 days of age, pups that were exposed to either the low dose or the high dose of nicotine both prenatally and postnatally had significantly lower hepatic MDA levels than those in group C, although pups exposed to the high dose of nicotine had a significant increase in the incidence and severity of focal necrosis and an increase, although not significantly, in the incidence and severity of confluent necrosis. The decrease in hepatic MDA level in the presence of mild focal necrosis is also seen in the study of nonpregnant rats (15, 16). In nonpregnant rats, a biphasic response, which is dose dependent, has been found in hepatic MDA levels (16). Rats exposed to 54 μM nicotine had a decrease in hepatic MDA levels and an increase in mild focal necrosis, although not significantly. However, those exposed to 108 μM nicotine had an increase in hepatic MDA levels (16) and, concurrently, a moderate degree of liver damage was noted (15). Interestingly, although there were no differences in hepatic MDA levels among groups at birth, pups that were exposed prenatally to either the low or high dose of nicotine have lower MDA levels when they were 10 days old when compared with those in group C even though there was no further exposure of the pups to the alkaloid after birth. We cannot explain the reason why hepatic necrosis did not cause an increase in MDA level in our rat pups. We suspect that since our liver homogenates contain both necrotic and non-necrotic hepatocytes, the increase in mild focal necrosis at birth may not be severe enough to cause a measurable increase in MDA levels. We also suspect that at 10 days of age, the lower hepatic MDA levels in pups exposed to nicotine *in utero* may be a consequence of a relatively more chronic damage rather than acute damage to the liver. Indeed, it has been shown that cigarette smoking can acutely increase MDA level but decline thereafter even though cigarette smoking continues (24), indicating that MDA increases only in acute damage but decreases in chronic injury.

Unlike MDA levels, decrease in SOD activities in our pups reflects those of histological changes. This suggests that an imbalance in oxidant-antioxidant mechanism is involved in the liver damage observed. Various chemicals, including nicotine, have been shown to cause cellular damage by affecting the cellular antioxidant defense systems (9, 25, 26). Pathogenesis of atherosclerosis in nicotine-administered rats is associated with a decrease in SOD activity (9). Addition of SOD enzyme to nicotine-treated tissues *in vitro* has been shown to dampen the effects of lipid peroxidation (10, 11, 23). Our data showed that SOD levels in all our rat pups were depressed during nicotine exposure. Interestingly, when rat pups that were exposed to nicotine *in utero* discontinued their nicotine exposure after birth, their

SOD levels at 10 days of age were similar to those of control pups not exposed to any nicotine throughout the study. This finding supports our speculation that the oxidative stress-induced hepatic damage caused by nicotine is a result of depressed SOD levels.

In conclusion, nicotine-induced hepatic focal necrosis *in utero* is still evident at 10 days of age, even after withdrawal from the nicotine exposure at birth. Incidence and severity of focal necrosis increases with further postnatal exposure. Postnatal exposure to nicotine for 10 days induces only mild focal and confluent necrosis, but not significantly. Oxidative stress is implicated in the nicotine-induced hepatic necrosis. This study shows that the cause of the oxidative stress is the result of a decrease in SOD activity. The role of lipid peroxidation in nicotine-induced liver damage is not as clear.

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