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ORIGINAL ARTICLE

The Effects of Nicotine on Foetal Loss, Postnatal Growth and Plasma Oestrogen and Progesterone Levels in Rats

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ABSTRACT

Introduction: The present study aims to investigate the effects of nicotine on foetal loss, postnatal growth and corresponding levels of oestrogen and progesterone in pregnant rats. **Methods:** Subcutaneous injection of nicotine tartrate (7.5 mg/kg/day) was administered to groups of pregnant rats; with treatment scheduled from day 1 through day 5, day 5 through day 9 or day 1 through day 9 of pregnancy. On day 10 of pregnancy, laparotomy was performed to count the number of blastocyst implantation sites. During parturition, the number of viable pups was recounted and statistically compared with the controls. One group of rats which received nicotine from day 1 through day 9 of pregnancy was sacrificed on day 16 of pregnancy, and circulating levels of oestrogen and progesterone were measured. Upon delivery, the birth weight of the pups was measured, and their weights were recorded until weaning. **Results:** There was a significant increase in foetal loss particularly in rats which received nicotine from day 5 through day 9 and from day 1 through day 9 of pregnancy. There was also significantly lower birth weight of pups in all groups; however, this pattern did not continue until weaning. Plasma oestrogen level was significantly elevated with a significant decrease in the plasma progesterone level. **Conclusions:** Nicotine administration during pregnancy showed an increase in foetal loss with a corresponding increase in oestrogen and decrease in progesterone levels. Although the birth weight of the pups was low, there was catch-up growth in the pups.

KEYWORDS: Nicotine, pregnancy, foetal loss, oestrogen, progesterone

INTRODUCTION

Tobacco smoking has been identified as the leading cause of preventable deaths worldwide. This is a cause for alarm as the number of smokers has been increasing worldwide especially in the developing countries and among women [1]. Smoking has been linked to pregnancy-related complications, cancer of the female reproductive organs and menstrual dysfunctions [2]. Nicotine in tobacco smoke has been shown to adversely affect female reproduction in almost all stages of reproductive processes including folliculogenesis, embryo transport, endometrial receptivity, steroidogenesis and even menopause [3]. Nicotine exposure during pregnancy increases the risk of foetal mortality and morbidity [4]. In humans, smoking and nicotine has been associated with an increase in the number of spontaneous abortions [5]. Foetal loss before 20 weeks of gestation was found to be 33% higher among smokers compared to that of non-smokers [6]. There was also an increased risk of abruptio placenta [7], per vaginal bleeding during pregnancy, premature rupture of membranes [8], preterm delivery [5] and stillbirth [4] among women who smoke during pregnancy. The increased risk of spontaneous abortion may be attributed to the effects of nicotine on the embryo transfer process, fertilization, implantation or placentation. Nicotine exposure during pregnancy has also been associated with low birth weight of the infants [9]. The low-birth weight of infants in nicotine-exposed mothers might be attributed to the effects of nicotine on fetal growth *in*

utero and on the placenta [9]. Nicotine has also been shown to disrupt the pattern of maternal plasma progesterone and oestrogen levels. Plasma progesterone level was reported to be significantly decreased during the first trimester in rats following nicotine administration [10]. However, conflicting reports exist on the effect of nicotine on the level of oestrogen; with some studies reporting a decrease [11, 12]; while others suggesting an increase [13, 14] in its level.

Exposure of nicotine during differing gestational periods yield differing outcomes [15]. In the current study, nicotine administration was scheduled in three different timeframes in relation to the decidualization of the endometrium, namely: period of endometrial sensitivity, period of endometrial growth and periods of both endometrial sensitivity and growth [16].

Another question is, although nicotine has been shown to have detrimental effects on foetal development, it is unclear as to during which stage of pregnancy that these effects are more severe. Subsequently, it is also unclear if foetuses exposed to nicotine in utero have normal development postnatally.

METHODS

Animals

Female Sprague-Dawley rats weighing 180-220g with regular 4-day oestrous cycle were used in this study. Animals had free access to laboratory chow and drinking water. The room temperature was kept at 25° C with a 12:12 hour light: dark cycle. The study design was approved by the Committee on Animal Research and Ethics, UiTM (approval code).

Experimental Design

Upon confirmation of pro-oestrous via a vaginal smearing, animals were randomized into eight groups and housed individually with a fertile male overnight. The presence of sperm in the vaginal smear the following morning confirmed positive mating and was considered as day 1 of pregnancy. Treatment was scheduled in three different timeframes as shown in Table 1, namely: period of endometrial sensitivity, which coincided with treatment from Day 1 to Day 5 of pregnancy, period of endometrial growth (from Day 5 to Day 9 of pregnancy) and both periods of endometrial sensitivity and growth together, which encompassed treatment from Day 1 to Day 9 of pregnancy [16]. Nicotine tartrate (Sigma-Aldrich, USA) solution with a concentration of 7.5 mg/kg was injected subcutaneously (sc) at the back of the neck [17] twice a day (at 0900 and 1600 hours) (Table 1). Control animals received equivalent volume of normal saline subcutaneously. On day 10 of pregnancy, the animals were anaesthetized with an intraperitoneal injection of a mixture of Ketamine (250 mg/5ml), Xylazine (20 mg) and Zoletil (50 mg) at a dose of 0.1 ml/100g body weight. Laparotomy was performed, the uterine horns were exposed, and the number of implantation sites counted. The site of incision was then sutured in layers using absorbable suture (Catgut 2.0) for the muscle and non-absorbable suture (Nylon 2.0) for the skin. The animals were allowed to recover and observed on a daily basis. On day 16 of pregnancy, animals in groups 4a and 4b were anaesthetised and blood was collected via cardiac puncture for oestrogen and progesterone estimation. The rats were then euthanised, and laparotomy was performed to remove the uterine horns. The foeto-placental unit was removed together with the overlying uterine tissue and weighed. The remaining groups of rats were allowed to go till term and daily inspection was done to see evidence of foetal expulsion and also to monitor the duration of pregnancy. Immediately following parturition, the number of pups delivered was counted and the birth weight of each pup was recorded. Foetal loss was calculated as the difference between the number of viable implantation sites on day 10 of pregnancy and the number of pups delivered. The weight of the pups was recorded every other day until weaning (post-natal day 21).

Table 1 Treatment schedules during pregnancy in control and nicotine-treated rats (n=6 for each group)

Group	Type of Treatment	Duration of Treatment
1a	Nicotine tartrate 7.5mg/kg/day	Days 1 to 5 of pregnancy
1b	0.9% normal saline	Days 1 to 5 of pregnancy
2a	Nicotine tartrate 7.5mg/kg/day	Days 5 to 9 of pregnancy
2b	0.9% normal saline	Days 5 to 9 of pregnancy
3a	Nicotine tartrate 7.5mg/kg/day	Days 1 to 9 of pregnancy
3b	0.9% normal saline	Days 1 to 9 of pregnancy
4a	Nicotine tartrate 7.5mg/kg/day	Days 1 to 9 of pregnancy
4b	0.9% normal saline	Days 1 to 9 of pregnancy (sacrificed on day 16 of pregnancy

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Hormonal Assay

Blood samples collected from animals in groups 4a and 4b were centrifuged at 3000 rpm for 15 minutes and stored at -80ºC. Oestrogen and progesterone estimation in plasma was done using the automated electrochemiluminescence immunoassay (ECLIA) technique (Roche Elecsys, Switzerland).

Statistical Analysis

Normality of the data distribution was assessed using Kolmogorov-Smirnov test. Normally distributed data were analyzed using t-test and the data were expressed as mean ± SEM. Skewed data were analyzed using Mann-Whitney test and expressed as median (interquartile range, IQR). Chi square test was used to test categorical variable. Statistical significance was defined as $p<0.05$ and all the statistical analyses were performed using statistical test contained in the SPSS 19.0 software.

RESULTS

There was no significant difference in the duration of pregnancy between the control and all the nicotine-treated groups (Table 2).

Table 2 Duration of pregnancy (days) in control and nicotine-treated groups

Groups	Pregnancy duration (days)		
	1a and 1b, median (IQR)	2a and 2b, median (IQR)	3a and 3b, median (IQR)
Nicotine-treated	23.00 (0)	23.00 (1)	23.00 (0)
Control	23.00 (0)	23.00 (0)	23.00 (0)
Z	-0.114	-1.363	-0.935
P value	NS	NS	NS

NS = not significant

There was no significant difference in the number of blastocyst implantation sites on day 10 of pregnancy between the nicotine-treated groups and their respective controls (Table 3).

Table 3 Number of blastocyst implantation sites on day 10 of pregnancy in control and nicotine-treated groups.

Groups	Number of blastocyst implantation sites on day 10 of pregnancy		
	1a and 1b, mean ± (SEM)	2a and 2b, mean ± (SEM)	3a and 3b, mean ± (SEM)
Nicotine-treated	10.67 ± 0.57	10.83 ± 0.65	9.57 ± 0.53
Control	11.67 ± 0.56	11.67 ± 0.55	11.29 ± 0.61
t (df)	1.268 (10)	0.969 (10)	2.132 (12)
P value	NS	NS	NS

NS = Not significant

Foetal loss was significantly greater in the groups receiving nicotine from days 5 to 9 of pregnancy ($p<0.001$) and from days 1 to 9 of pregnancy ($p<0.001$) when compared to their respective controls. In the group that received nicotine from days 5 to 9 of pregnancy, fetal loss was 3.805 times greater than that in the control. Whereas the group that received nicotine from days 1 to 9 of pregnancy, the loss was 4.324 times greater than that in the control (Table 4).

Table 4 Number of viable pups and foetal loss at parturition in control and nicotine-treated groups

Groups	Number of blastocyst implantation sites	Number of pups at parturition	No. of foetal loss	OR (95% CI)	p value
1a	64	52	12	0.591	NS
1b	75	66	9	(0.231, 1.509)	
2a	65	41	24***	3.805	<0.001
2b	75	65	10	(1.651, 8.768)	
3a	67	41	26***	4.324	<0.001
3b	86	75	11	(1.940, 9.634)	

(Pearson chi-square, ***p<0.001)

On day 16 of pregnancy, significant foetal loss was evident in animals treated with nicotine when compared to that in its control (p value = 0.008). The loss was 2.86 times higher [OR=2.862 (95%=1.294,6.332)] (Table 5).

Table 5 Number of viable pups and foetal loss at day 16 of pregnancy in control and nicotine-treated groups

	Day 10 of pregnancy	Day 16 of pregnancy			
Groups	Number of healthy foetuses	Number of healthy foetuses	Number of foetal loss	OR (95% CI)	p value
4a	54	29	25**	2.86	0.008**
4b	68	57	11	(1.294, 6.332)	

The weight of the foeto-placental unit on day 16 of pregnancy was significantly lower in nicotine-treated rats when compared to that in gestation-matched controls ($p<0.001$; Table 6).

Table 6 Weight of the foeto-placental unit/swelling (g) on day 16 of pregnancy in groups 4a and 4b

Group	Weight of the foeto-placental unit/swelling (g), mean ±SEM	t (df)	p value
Nicotine-treated	0.99 ± 0.02***	3.911	<0.001
Control	1.08 ± 0.01	(119)	

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Birth weight of the pups in all the three nicotine-treated groups was significantly lower when compared to their respective controls. However, this pattern of lower weight did not continue until weaning. On day 21 post-natal, the weight of the pups in all three nicotine-treated groups was not different from their respective controls as shown in Table 7.

Table 7 Weight of the pups (g) at birth and at day 21 post-natal in control and nicotine-treated group

Groups	Pups weight (g)					
	1a and 1b (median, IQR)		2a and 2b (median, IQR)		3a and 3b, median (IQR)	
	At Birth	Day 21	At Birth	Day 21	At Birth	Day 21
Control	7.01 (0.69)	33.08 (1.83)	6.97 (0.91)	33.02 (1.90)	6.90 (0.87)	31.80 (1.59)
Nicotine-treated	6.41 (0.61)***	32.00 (3.00)	6.45 (1.35)***	33.00 (3.50)	6.35 (0.66)***	31.00 (5.50)
Z	-6.279	-1.870	-3.669	-0.318	-5.493	-1.679
p value	<0.001	NS	<0.001	NS	<0.001	NS

Compared to that in the controls, plasma oestrogen level on day 16 of pregnancy was significantly higher in animals treated with nicotine from day 1 to 9 of pregnancy ($p<0.01$), whereas the progesterone level was significantly lower in nicotine-treated animals when compared to that in the control (Table 8).

Table 8 Plasma oestrogen (pmol/L) and progesterone (nmol/L) levels on day 16 of pregnancy in rats given nicotine from day 1 to day 9 of pregnancy

Group	Plasma level of oestrogen (pmol/L), mean ± SEM	Plasma level of progesterone (nmol/L), mean ± SEM
Nicotine-treated	103.64 ± 6.24**	341.27 ± 25.15*
Control	77.33 ± 5.37	419.00 ± 26.14
t (df)	-3.197 (26)	2.143 (16)
p value	<0.01	<0.05

DISCUSSION

The present study demonstrates that nicotine significantly: 1) increased fetal loss, particularly when exposed to nicotine from day 5 to day 9 of pregnancy and day 1 to day 9 of pregnancy and 2) lowered the birth weight of pups. The detrimental effect of nicotine is usually dose-dependent and depends on the duration of exposure [18]. These findings may be attributed to nicotine's detrimental action on the growth of the placenta. Nicotine exposure in pregnant rats was shown to cause vasoconstriction of uterine arteries which disrupted the uterine circulation, resulting in reduction of blood flow [19]. A sufficient flow to the uteroplacental vessels is very important to achieve a normal pregnancy outcome. Haemodynamic changes in the maternal uterine circulation are characterized by a significant decrease in the resistance of the uterine vessels, which in turn, is due to vessel growth and vasodilation [20]. It was reported in rats that intrauterine growth restriction was associated with altered utero-placental perfusion [21]. These may explain the increased number of foetal loss during parturition (Table 4) and the decreased weight of the uteroplacental swelling on day 16 of pregnancy (Table 6). The number of foetal loss is significantly higher in groups treated with nicotine from day 5 to day 9 of pregnancy when compared to that of groups treated with nicotine from day 1 to day 5 of pregnancy. This finding may be due to the fact that in rats, implantation and thus growth of placental layers and structures only begin after day 5 of pregnancy. Thus, the decidualization of stroma following implantation only begin on day 4 or day 5 of pregnancy [22].

The present study also showed that nicotine reduces the birth weight of the pups, possibly via its detrimental effect on the placenta, and subsequently fetal growth is restricted. This low birth weight pups may also be attributed to the action of nicotine on maternal food intake. The exact mechanism of low maternal food intake is unknown but it was shown that nicotine activated the pro-opiomelanocortin (POMC) neurons which is responsible for stimulating the satiety center [23]. Nicotine administration has also been shown to increase the level of corticotropin-releasing hormone (CRH) [24], which will also stimulate the satiety center. It leads to decreased food intake and the subsequent low body weight may contribute to low birth weight infants.

Although the birth weight of pups significantly decreases following nicotine administration, there was a catch-up growth in early infancy as shown in the postnatal weight at day 21. This catch-up growth may be due to the effect of nicotine on metabolic changes. Exposure to nicotine during the perinatal stage was shown to cause an increase in body weight and fat mass in adult male rats despite having no change in the food intake [25]. This was attributed to the effect of nicotine in altering early adipogenesis [25]. Several epidemiological studies [26, 27] have reported the association between maternal smoking and increased weight of the offsprings during childhood with an

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increased risk of developing obesity and hypertension in later life [28]. However, the exact mechanism in human was unknown.

Apart from its detrimental effects on the placenta, nicotine may also directly affect the fetus. This possibility was based on the study by Zhao and Reece [29] who showed that nicotine caused various developmental abnormalities. They found that murine embryo cultures treated with nicotine resulted in abnormalities such as opened neural tube in the anterior regions with opened forebrain and hindbrain, both of which are incompatible with life [29]. There was also developmental retardation as evidenced by shorter crown/rump length [29]. They attributed these findings to the increased level of intracellular Ca2+ and oxidative stress following nicotine treatment [29].

In the current study, nicotine significantly increased the level of maternal plasma oestrogen (Table 8). An increase in the level of circulating maternal oestrogen may have also contributed to the increased foetal loss. It was reported by Bartholomeusz et al [30] and Matsuura et al [31] that high dosage of oestrogen given to pregnant rats resulted in reduced survival of the foetus. They reported that even minor elevation of maternal oestrogen during mid-gestation may elicit foetal death. The exact mechanism by which this foetal loss occurs due to increased oestrogen level is still unclear but it may be attributed to the effects of oestrogen on the reduced density of the foetal blood vessels [30]. Oestrogen also has a direct effect on the uterus. It is known that oestrogen promotes uterine contraction [32] as seen during parturition. The increased uterine contraction before parturition may lead to premature foetal expulsion and loss.

It is tempting to suggest that the increased oestrogen coupled with low progesterone level found in this study (Table 8) may explain the high foetal loss. Progesterone is an essential hormone to maintain uterine function during pregnancy. Nicotine was shown to distort the ovarian cytoarchitecture which may affect the progesterone level during early pregnancy [10]. Progesterone also promotes myometrial relaxation during early pregnancy, and withdrawal of this hormone has been shown to initiate parturition [33]. Decreased maternal progesterone has also been shown to retard placental and foetal growth, especially in the placental basal zone, and subsequently affect the survival of the fetus [34].

This interplay of oestrogen and progesterone is also important in decidualization in the rats, and formation of the maternal part of the placenta [35]. It was shown that the combination of decreased level of progesterone with increased level of oestrogen may compromise the growth of the decidua cells [36]. The compromised decidualization will affect the growth and development of the foetus and may lead to an increased foetal loss as well as lower birth weight of pups.

CONCLUSION

The foetal loss and low foetal birth weight observed in the present study appears to be partly due to altered plasma levels of oestrogen and progesterone following nicotine administration.

Conflicts of Interest

Authors declare none.

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