Detection of cotinine in neonate meconium as a marker for nicotine exposure *in utero*

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كشف الكوتينين في عِقْي الولدان كواسم للتعرض للنيكوتين داخل الرحم

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الخلاصة: تم قياس مستوى الكوتينين في عِقْي الولدان كواسم يدلُّ على تعرضهم قبل ولادتهم للنيكوتين من تدخين أمهاتهم للتبغ. فقد قسمت الوالدات اللواتي أدخلن إلى مستشفى الولادة في الإسكندرية، مصر إلى ثلاث مجموعات: 10 مدخنات فاعلات، 10 مدخنات بالتعرض لدخان المدخنين الاخرين، 10 لم يتعرضن للتبغ أثناء حملهن. وقد جمعت عينات من لعاب وبول الأمهات وعِقْي ولدانهن في اليوم الأول. وقد وجد أن المستوى الوسطي للكوتينين في بول الأمهات يختلف لدى المجموعات الثلاث لدى قياسه بالمقايسة المناعية الإشعاعية. كما وجد مثل هذا الاختلاف في المستوى الوسطي للكوتينين في اللعاب والعقي. وكان هناك ارتباط إيجابي هام بين مستويات الكوتينين في العقي وكل من مستويات الكوتينين في لعاب وبول الأمهات. وهكذا يعد العِقْي واسماً بيولوجيا مثالياً لاختبار التعرض المباشر للجنين لدخان التبغ في فترة حياة الوليدان.

ABSTRACT Neonate meconium cotinine level was evaluated as a marker of prenatal exposure to nicotine from tobacco smoking by mothers. Mothers admitted to a maternity hospital in Alexandria, Egypt, were divided into 3 groups: 10 active smokers, 10 passive smokers and 10 with no tobacco exposure during pregnancy. Urine and saliva samples were collected from mothers and first-day meconium samples from their neonates. Mean maternal urinary cotinine levels, measured using radioimmunoassay, differed significantly between the 3 groups, as did mean salivary cotinine and mean cotinine levels in meconium. There was a significant positive correlation between cotinine levels in meconium and both maternal urinary and salivary cotinine levels. Meconium is an ideal biological marker for testing direct fetal exposure to tobacco smoke in the neonatal period.

Détection de la cotinine dans le méconium du nouveau-né comme marqueur de l'exposition à la nicotine *in utero*

RESUME Le taux de cotinine dans le méconium du nouveau-né a été évalué en tant que marqueur de l'exposition prénatale à la nicotine du fait du tabagisme de la mère. Les mères admises dans une maternité à Alexandrie (Egypte) ont été réparties en trois groupes : 10 fumeuses actives, 10 fumeuses passives et 10 femmes qui n'avaient pas été exposées au tabac pendant la grossesse. Des échantillons d'urine et de salive ont été recueillis chez les mères et des prélèvements de méconium du premier jour ont été effectués chez leurs nouveau-nés. Le taux moyen de cotinine urinaire de la mère, mesuré par radio-immunodosage, différait significativement entre les trois groupes, tout comme le taux moyen de cotinine salivaire, ainsi que le taux moyen de cotinine dans le méconium. Il y avait une corrélation positive significative entre le taux de cotinine dans le méconium et le taux de cotinine salivaire et urinaire chez la mère. Le méconium est un marqueur biologique idéal pour déceler l'exposition directe du fœtus au tabac dans la période néonatale.

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Introduction

There is no doubt that smoking during pregnancy is hazardous to both the mother and baby. The harmful effects of maternal smoking have been well documented. The relation between maternal cigarette smoking and adverse pregnancy outcomes include abruptio placenta, bleeding during pregnancy, premature rupture of membranes, fetal death, neonatal mortality, and deficits in growth, intellectual and emotional development and in behaviour [*1*]. The majority of epidemiological studies have found no association between cigarette smoking and congenital malformations [*2*]. However, one study suggested that mothers aged 35 years and older who smoke may have a higher risk of delivering infants with minor malformations [*3*].

The risk of passive smoking or environmental exposure to tobacco smoke has been a major concern in the past two decades. It has been suggested that passive maternal smoking may be associated with lower score on tests of neurodevelopment as a result of long-term neurotoxicity [*4*]. This could be explained by the fact that fetal and neonatal levels of carboxyhaemoglobin are generally higher than maternal concentrations because of a higher affinity of fetal haemoglobin for carbon monoxide [*4*].

Cotinine is a more specific indicator for the inhalation of tobacco smoke, i.e. nicotine, compared with other indicators such as carboxyhaemoglobin. Carboxyhaemoglobin is a non-specific indicator as it is formed on exposure to carbon monoxide, which is widely distributed as an air pollutant and is also endogenously produced. Cotinine is the major metabolite of nicotine [*5*]. It is considered a better marker of long-term exposure to tobacco smoke than the parent alkaloid, since it is endogenously produced only through an oxidative metabolism of nicotine in the body [*6*]. It is also more easily identified, since its concentrations are higher in both blood and urine. This is due to its longer plasma half-life (30 hours) as well as a more protracted excretion rate [*7,8*].

The aims of the study were to: evaluate the use of neonate meconium as a marker of prenatal exposure to nicotine from tobacco smoking by the mother; to study the correlation between maternal urinary and salivary cotinine levels and the levels found in the neonate meconium; and to study the effect of nicotine on birth weight.

Methods

The study was carried out from 1 July 1999 to 31 January 2000 on 30 consecutive mothers and their neonates admitted to El-Shatby Maternity University Hospital. All mothers were interviewed and a full history was taken with emphasis on history of smoking during the last pregnancy. Neonates were weighed and clinically examined for any congenital anomalies. The mothers were subdivided into 3 subgroups according to smoking habits: 10 mothers with a history of active smoking during the pregnancy; 10 mothers with a history of passive smoking during the pregnancy; and 10 mothers with no exposure to smoking during the pregnancy.

Urine and saliva were collected from mothers as well as first day meconium from their neonates. Consent was obtained from mothers before urine collection. Urine was collected from mothers at the time of delivery by a catheter to avoid contamination of urine with blood. A 10–20 mL sample of urine was placed in a plastic container and frozen at –15 °C to –20 °C until the time of assay. A saliva sample of 2–3 mL was collected (unstimulated) from mothers shortly after labour and placed in

plastic test-tubes and deep frozen until the time of assay. First-day meconium was collected straight from the diaper of the neonates using a spatula and deep frozen in plastic tubes until the time of extraction.

Cotinine extraction and assay

The urine and saliva samples of the mothers were centrifuged and the supernatants were assayed directly by radioimmunoassay (RIA) using a double antibody nicotine metabolite procedure (Diagnostic Products Corporation, Los Angeles, USA) [*9*]. The procedure is a liquid-phase RIA in which cotinine labelled with iodine 125 competes for antibody sites for a fixed time with cotinine (and other nicotine metabolites) in the patient's sample. After incubation for a fixed time, separation of bound from free parts is achieved by the polyethylene glycol (PEG)-accelerated double antibody method. Finally, the antibody-bound fraction is precipitated and counted. The patient sample concentration is read from a calibration curve.

The reproducibility of this method was examined on urinary samples selected to represent a range of 3 different cotinine levels. Results revealed a coefficient of variation of 5.3%–9.4% for intra-assay (within-run) and 4.3%–6.8% for inter-assay (run-to-run) results.

After thawing, meconium samples of 0.5 to 1.0 g were weighed and vortexed rigorously in 5 mL of methanol. After centrifugation at 1500 × g, the supernatant was dried in a 40 °C water bath using a stream of nitrogen [*10*]. The sample was then reconstituted with 1.0 mL of methanol and 50 µL was taken off and assayed using the same RIA method [*9*].

Statistical analysis

Statistical analysis was made using *SPSS*, version 8 [*11*]. Statistical tests used were the mean and standard deviation (SD), *F*-test (ANOVA), Pearson correlation coefficient, Spearman correlation coefficient and Scheffe test. A *P*-value of 5% was used as the level of significance.

Results

Maternal data

The age of the active smoker group ranged from 20 to 32 years with a mean of 27.8 ± 3.7 years. The age of passive smokers group was lower, ranging from 18 to 35 years with a mean of 26.3 ± 5.4 years. In the non-exposed group, the age ranged from 20 to 29 years and the mean was 25.0 ± 2.9 years. There was no significant difference between the mean ages of the 3 groups ($F = 1.15$).

The number of cigarettes smoked per day by the mothers of the active smokers group ranged from 2–18 cigarettes with a mean of 11.7 ± 5.3 cigarettes.

Neonatal data

Using the Scheffe test, neonates whose mothers were active smokers had a lower mean birth weight than those whose mothers were passively exposed to smoke (mean difference 200.0 ± 82.5 g). This difference was not significant, however. Neonates whose mothers smoked had a significantly lower weight than those with non-smoking mothers (mean difference 490.0 ± 82.5 g) ($P < 0.001$) (Table 1). The mean birth weight of neonates in the passive smoking group was less than those in the non-smoking group (mean difference 290.0 ± 82.5 g), and this was also significant ($P < 0.05$).

Laboratory data

The present study revealed an overlap between the levels of maternal urinary cotinine in active and passive smokers. The highest level was detected in the active

Table 1 **Mean difference in birth weight in relation to maternal smoking habit**

Groups compared	Difference in birth weight (g) Mean	SE	*P*-value
Active (n = 10) versus passive (n = 10) smokers	–200.0	82.5	0.07
Active (n = 10) versus non-smokers (n = 10)	–490.0	82.5	< 0.001
Passive (n = 10) versus non-smokers (n = 10)	–290.0	82.5	0.006

SE = standard error.

smoking group, ranging from 1390 to 22 300 ng/mL, with a mean of 16 110 ± 10 851 ng/mL (Table 2). In passive smoking mothers, the cotinine level ranged from 79.3 to 14 385 ng/mL, with a mean level of 3096 ± 5783 ng/mL. The lowest level was detected in the urine of non-smoking mothers where it ranged from 0 ng/mL (non-detectable) to 122.5 ng/mL with a mean of 75.1 ± 42.2 ng/mL. There was a significant difference in the urinary cotinine levels across the 3 groups (F = 14.41, $P < 0.01$).

There was a significant difference in the mean cotinine levels in mothers' saliva among the 3 groups (91.8 ± 101.6 ng/mL, 13.7 ± 19.2 ng/mL and 1.1 ± 2.0 ng/mL in active, passive and non-smokers respectively) (F = 6.79, P = 0.01) (Table 3).

There was a significant difference between the mean cotinine levels in the saliva of active smokers and passive smokers using the Scheffe test (mean difference 78.2 ± 26.7 ng/mL). There was also a significant mean difference between the salivary cotinine levels of active smokers and non-smokers (mean difference 90.7 ± 26.7 ng/mL) (Table 4).

The Pearson correlation test showed a positive correlation between the cotinine levels in mothers' saliva and mothers' urine (r = 0.582, where P = 0.01) (Figure 1).

Table 2 **Maternal urinary cotinine levels in relation to maternal smoking habit**

Smoking group	Urinary cotinine level (ng/mL) Range	Mean	SD
Active smokers (n = 10)	1390–22 300	16 110.0	10 851.3
Passive smokers (n = 10)	79.3–14 385	3095.9	5782.8
Non-smokers (n = 10)	0–122.5	75.1	42.2
F-test			F=14.41, P< 0.01

SD = standard deviation.

Table 3 **Maternal salivary cotinine levels in relation to maternal smoking habit**

Smoking group	Salivary cotinine level (ng/mL) Range	Mean	SD
Active smokers (n = 10)	1.7–250.0	91.6	101.6
Passive smokers (n = 10)	0–65.0	13.7	19.2
Non-smokers (n = 10)	0–6.5	1.1	2.0
F-test		F = 6.79, P < 0.01	

SD = standard deviation.

Table 5 shows that in the active smoker group, the cotinine level in meconium ranged from 232 to 700 ng/mL with a mean of 367.2 ± 143.7 ng/mL. In the passive smokers group, it ranged from 148 to 350 ng/mL with a mean of 263.4 ± 52.5 ng/mL, while in the non-smokers group it ranged from 153 to 213 ng/mL with a mean of 185.0 ± 24.2 ng/mL. Within the 3 groups, there was a significant difference in the cotinine levels in meconium (F = 10.45, P = 0.01).

There was a positive correlation between the cotinine levels in meconium and in mothers' urine (r = 0.688, P = 0.01) (Figure 2). There was also a significant positive correlation between the cotinine levels in meconium and in mothers' saliva (r = 0.784, P = 0.01) (Figure 3).

The Pearson correlation test showed a significant negative correlation between the cotinine levels in maternal urine and the neonatal birth weight (r = 0.546, P = 0.01) (Figure 4). The study also showed a significant negative correlation between the cotinine level in meconium and birth weight (r = 0.497, P = 0.01) (Figure 5).

Discussion

Cigarettes are the most common non-medicinal drugs used by pregnant women [*12*]. The present study was carried out to evaluate meconium as an alternative biological marker for the detection of the nicotine metabolite cotinine.

Thirty mothers and their neonates were chosen for this study. The small sample size is due to the small number of mothers who confessed that they smoke, as smoking by women is culturally unacceptable in Egypt. The babies were all full term, as gestationally premature babies were ex-

Table 4 **Mean difference in maternal salivary cotinine levels in relation to maternal smoking habit**

Groups compared	Difference in salivary cotinine level (ng/mL) Mean	SE	P-value
Active (n = 10) versus passive (n = 10) smokers	78.15	26.7	0.024
Active (n = 10) versus non-smokers (n = 10)	90.74	26.7	0.008
Passive (n = 10) versus non-smokers (n = 10)	12.59	26.7	0.895

SE = standard error.

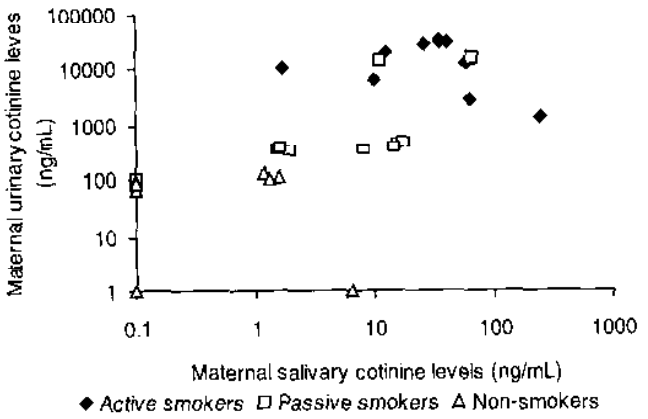

Figure 1 **Correlation between cotinine levels in maternal saliva and maternal urine**

cluded from the study. This was to limit any confusion between the effects of tobacco smoking by the mother during pregnancy and the effects of prematurity.

The maternal data showed that there was no significant difference between the mean age of active smokers, passive smokers and non-smokers. That both smoking and exposure to passive smoking are not confined to a specific age group highlights the general lack of awareness regarding the effect of smoking during pregnancy.

The mean number of cigarettes smoked by the active smoker group was 11.7 per day. This is almost identical to the study of Eliopoulos et al. who found the mean number of cigarettes smoked per day by pregnant smokers was 11.8 [*13*].

In the present study using the Scheffe test, the lowest birth weight was recorded in neonates of active smoking mothers. This is to be expected, as it has been reported that for every 10 cigarettes smoked by mothers, the risk of delivering a low birth weight for gestational age infant increases by a factor of 1.51. The effect is dose dependent, and is not the result of a shortened gestation period, but is due to fetal hypoxia arising from decreased uteroplacental perfusion [*14*]. Neonates of passive smoking mothers also had a significantly lower birth weight than babies of non-smokers in our study, indicating the dangers of passive smoking to neonates.

Table 5 **Cotinine levels in neonate meconium in relation to maternal smoking habit**

Smoking group	Meconium cotinine level (ng/mL)		
	Range	Mean	SD
Active smokers ($n = 10$)	232–700	367.2	143.7
Passive smokers ($n = 10$)	148–350	263.4	52.5
Non-smokers ($n = 10$)	153–213	185.0	24.2
F-test		$F = 10.45$, $P = 0.01$	

SD – standard deviation.

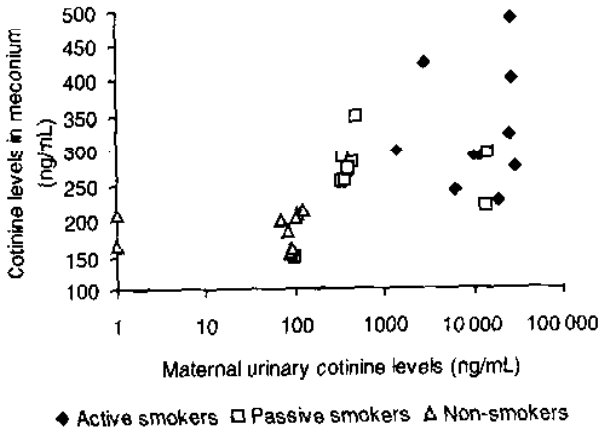

Figure 2 **Correlation between cotinine levels in maternal urine and neonate meconium**

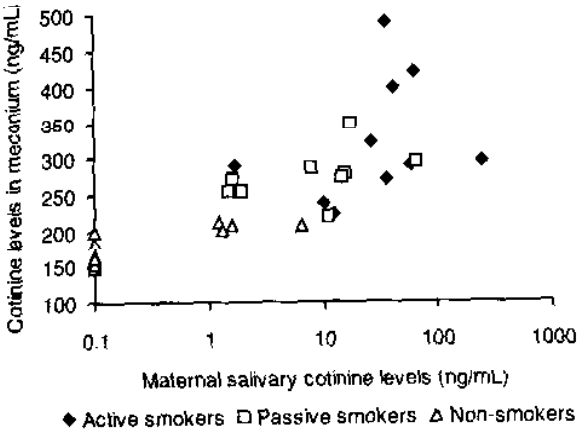

Figure 3 **Correlation between cotinine levels in neonate meconium and maternal saliva**

Cotinine was chosen for detection of smoking as it is the main metabolite of nicotine (70%) [*15,16*]. Its long plasma half-life (30 hours) and protracted excretion rate mean that concentrations in both blood and urine are high [*7,8*]. The present study showed that cotinine was detected in the urine and saliva of active, passive and non-smoking groups, ranging from a mean of 16 110 ng/ml in active smoking mothers to

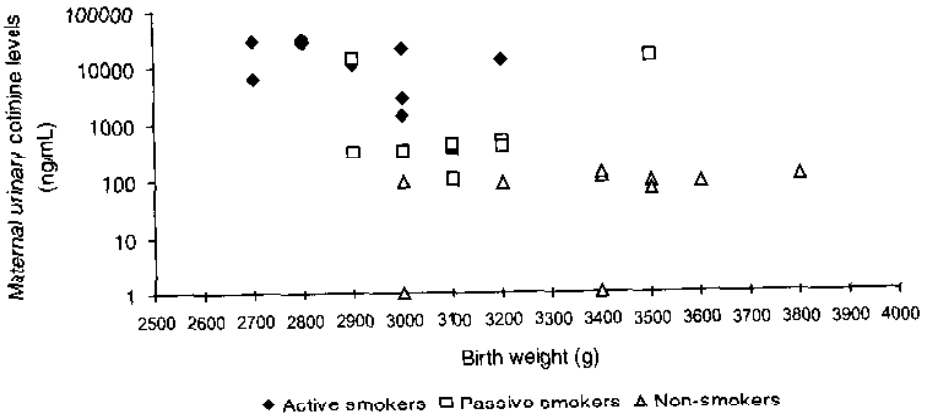

Figure 4 **Correlation between cotinine levels in maternal urine and birth weight**

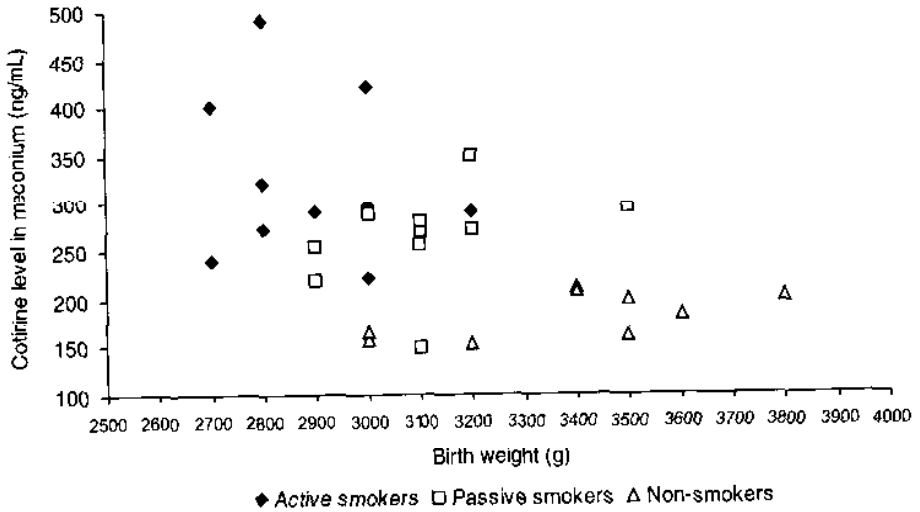

Figure 5 **Correlation between cotinine levels in neonate meconium and birth weight**

3096 ng/mL and 75.1 ng/mL in passive and non-smoking mothers respectively. There was an overlap between the range of maternal urinary cotinine levels in active and passive smokers, which agrees with the study of Wald et al. [*17*]. This may be attributed to variations in exposure, as an active smoking mother may be a light smoker

while a passive smoking mother may be heavily exposed to smoking. The study also revealed a slight overlap between urinary cotinine levels of passive and non-smokers. This is expected if the exposure is slight in the passive group. The presence of cotinine in the urine of non-smokers is not surprising as complete avoidance of cigarette smoke in the environment is almost impossible. Also, there are small amounts of nicotine in common foods such as potatoes, eggplant and tea [*18*].

There were significant differences between the 3 groups in both the mean cotinine levels in mothers' urine and the mean cotinine levels in mothers' saliva. Using the Pearson correlation test, we found a significant positive correlation between cotinine levels in urine and saliva ($r = 0.582$, $P = 0.01$). So mother's saliva could serve as an alternative to urine as a marker for detection of tobacco smoking. It is easier to obtain and difficult to adulterate.

The presence of cotinine in meconium was used as a direct indicator of fetal exposure to tobacco smoking in the neonatal period as cotinine is mainly deposited in meconium through bile secretion and to a lesser extent by fetal swallowing of amniotic fluid containing fetal urine [*15,16*]. The study revealed a significant difference in the cotinine levels of meconium between the 3 groups ($F = 10.45$, $P = 0.01$).

The mean concentration of cotinine in the meconium of neonates of active smokers was significantly higher than in neonates of passive and of non-smokers. But the mean concentration of cotinine in the meconium of neonates of passive smokers was not significantly higher than that of neonates of non-smokers.

Ostrea et al. used RIA to measure nicotine metabolites in the meconium which was extracted by vortex-mixing (0.5–0.6 g) meconium with 10 mL distilled water and 1 mL concentrated HCl, then filtering the homogenate through glass wool and using the centrifuged (9770 × g for 10 minutes) supernatant for the assay. They reported much lower cotinine levels ranging from 10.9 ng/mL in neonates of non-smokers to 54.6 ng/mL in neonates of heavy smokers [*19*], whereas the range of meconium cotinine levels in the present study were from 153 ng/mL in neonates of non-smokers to 700 ng/mL in neonates of active smokers. These differences in the levels could be attributed to either the difference in the extraction method used, as the extraction procedure substantially affects the outcome of the analysis [*20*], or to environmental tobacco smoke exposure in the present study.

As regards babies' birth weight, the Pearson correlation test showed a significant negative correlation between both maternal urinary cotinine levels and neonatal meconium cotinine levels and babies' birth weight. This confirms other studies showing that tobacco smoking leads to low birth weight and that fetal hypoxia increases with increasing exposure of the mother to tobacco smoke [*14*].

Conclusion

Cotinine was detected in maternal urine and saliva and these two biological fluids are good markers for tobacco smoking. Similarly, its presence in meconium proved that meconium is an ideal biological marker for testing direct fetal exposure to tobacco smoking in the neonatal period. This is important as it may throw light on the cause of a neonate with low birth weight. Added to this, collection of meconium is easy and non-invasive.

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