

Cytological evaluation of the nasal mucosa in neonates exposed to tobacco smoke during fetal life

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Abstract

OBJECTIVES: The objective of this study is to assess the cytological picture of the nasal mucosa of neonates born to mothers who are active smokers, passive smokers and non-smokers.

METHODS: A prospective study was conducted in a group of 86 neonates born between 23 and 41 weeks of gestation. The assignation of neonates to one of the three aforementioned groups was based on a questionnaire concerning exposure to tobacco smoke, and on the concentration of cotinine in maternal urine. A cytological examination was performed using exfoliative cytology with a semi-quantitative evaluation of the cells present in the specimens. Hematological summation equipment was used to assess the number of neutrophils, eosinophils, columnar, goblet, basal and squamous cells out of 500 cells counted. The number of specific cells was expressed as a percentage and a cytogram was created.

RESULTS: The most common type of cytogram contained neutrophils, columnar cells, and squamous cells. No significant differences were observed between the subgroups. Similarly, there was no correlation between the median of each type of cell and the cotinine concentration in the mothers' urine.

CONCLUSION: Active and passive smoking during pregnancy do not influence the cytological picture of the nasal mucosa of neonates.

Abbreviations:

- HPLC-DAD - high performance liquid chromatography with diode detection
- LOD - limit of detection
- LOQ - limit of quantitation

INTRODUCTION

There is strong evidence showing that exposure to environmental tobacco smoke increases the risk of upper and lower respiratory illnesses in children such as: bronchitis, pneumonia, wheezing, asthma, acute and recurrent otitis media. It also promotes the development of allergic diseases of the respiratory tract (Jaakkola & Jaakkola 2002; Burke *et al.* 2012). An increase in the number of eosinophils and IgE positive cells has been observed in the nasal mucosa of children exposed to tobacco smoke in the domestic environment, similar to children with allergies (Vinke *et al.* 1999). Recently, more attention has been focused on the fetal and neonatal period of life. Some authors have reported that the initial sensitisation of the lymphatic system to environmental allergens may have already occurred during the final period of fetal life (Holt 1994; Holt 1995; Holt *et al.* 1995; Jenmalm & Bjorksten 1998).

There is also evidence that cigarette smoking during pregnancy affects the development of the immune system of the fetus resulting in its modified activity in the neonate (Noakes *et al.* 2003).

Chronic inhalation of cigarette smoke causes nasal respiratory epithelial hyperplasia, squamous metaplasia of the airway and nasal epithelium, mucus hypersecretion, mucus pooling, pulmonary connective tissue damage, as well as chronic airflow obstruction (Dye & Adler 1994).

Despite the fact that nicotine has a favorable impact on the synthesis of surfactant and accelerates functional maturity of the lungs (Lieberman *et al.* 1992), neonates of mothers who are smokers have a lowered state of lung function (decreased lung flow parameters) (Carlsen *et al.* 1997; Hoo *et al.* 1998; Bisgaard *et al.* 2009). Impaired respiratory function has been described in both term and preterm infants of smoking mothers, suggesting that maternal smoking alters airway and alveolar development during fetal life (Hoo *et al.* 1998).

Some toxins of tobacco smoke are transferred from the mother to the fetus through the placenta (Alvarez *et al.* 2013; Ebrahim & Ashtarinezhad 2015). Significant concentrations of nicotine and its metabolites are detected in the amniotic fluid of pregnant women who are smokers (Rühle *et al.* 1995; Jauniaux *et al.* 1999; Benowitz *et al.* 2009; Kohler *et al.* 2010).

It has been suggested that maternal smoking during pregnancy, and therefore exposure of the fetus to tobacco smoke, has an impact on the structure of the nasal mucosa of the neonate.

MATERIAL & METHODS

A prospective study was performed in a group of neonates born at the Anna Mazowiecka University Hospital in Warsaw who were patients of the Neonatal and Neonatal Intensive Care Department of the Medical University of Warsaw. Inclusion in the study was condi-

tionally based on maternal consent and the completion of a questionnaire concerning maternal smoking status. The study protocol was approved by the Medical University of Warsaw Ethics Committee.

Neonates from singleton pregnancies delivered between 23 and 41 weeks of gestation were included in the study. Ninety mothers consented to the study. Ultimately, 86 neonates were included. Four neonates were excluded from the study because the cytology samples obtained from their nasal cavities did not fulfil good staining criteria. Based on the questionnaire and the concentration of maternal urinary cotinine, the neonates were divided into three groups:

- neonates of mothers who were non-smokers, who were not exposed to environmental tobacco smoke (cotinine concentration in maternal urine <32.5 ng/ml)
- neonates of mothers who were passive smokers, non-smokers who were exposed to environmental tobacco smoke (cotinine concentration in maternal urine 32.5–200 ng/ml)
- neonates of mothers who were active smokers (cotinine concentration in maternal urine > 200 ng/ml).

There were 28 neonates in the group of mothers who were non-smokers, 29 in the group of mothers who were passive smokers, and 29 in the group of active smokers. Smears from the nasal cavities were taken from the neonates for cytological analysis in all three groups.

Measurement of cotinine

The concentration of cotinine was determined in maternal urine (5 ml) within the first day after delivery. The urine samples were frozen and stored at minus 80 °C until the concentration of cotinine was measured.

The concentration of urinary cotinine was determined using high performance liquid chromatography with diode detection (HPLC-DAD). Chromatography was performed with liquid-liquid extraction of the molecule from urine. Cotinine concentration was expressed as ng/ml of urine. The limit of detection (LOD) was 10 mg/ml. The limit of quantitation (LOQ) was set at the level of the lowest calibration on the calibration curve (32.5 ng/ml). The measurement of cotinine was performed by the Environmental Analysis Laboratory of the Chair and Department of Toxicology of the Karol Marcinkowski Medical University in Poznań.

Nasal mucosa cytogram method

Exfoliative cytology was used to assess the nasal mucosa. Samples were taken during the first three days of life from the nasal concha, with the aid of a loop of stainless wire with a rough surface and a size corresponding to that of the nasal cavity of the neonate. A smear was made on a microscope slide, fixed in 98% alcohol and stained within 48 hours with hematoxylin and eosin. A semi-quantitative method was used to assess the presence of cells in the smear. The method of hematological

summation was used to count 500 cells. The samples were examined under a light microscope at a magnification of 400. The evaluation was blind. The number of individual cells (epithelial: columnar, goblet, basal, squamous, as well as infiltrated cells: neutrophils and eosinophils) was expressed as a percentage of the total number and a cytogram was created based on this. A strict quantitative evaluation was not possible because of the difficulty of obtaining homogenous material which would have been distributed evenly within the smear.

Statistical methods

The results were analyzed using the STATISTICA 8.0 program. A p value below 0.05 was considered as statistically significant. The results were presented in the form of a cytogram, and in view of the non-normal distribution of the data the median was used as the quantitative descriptor.

A comparison of the significance of the differences in the parameter between the groups, the number of specific cells expressed as a percentage, was made using the non-parametric Mann-Whitney test for non-normal distributions.

The method of cell profiles in the cytogram i.e. determination of the dominant type of cell (contributing to more than 50% of the total) showed the following profiles:

Neutrophilic >50% neutrophils in the cytological picture; Columnar >50% columnar cells in the cytological picture; Basal >50% basal cells in the cytological picture; Squamous >50% squamous cells in the cytological picture; Mixed – No dominant cell types

The chi-square test with the Yates' correction was applied for the comparison of the groups of children described above when the groups were small (<5).

RESULTS

Four mothers underestimated their exposure to tobacco smoke. One neonate whose mother had specified a non-smoker status with non-exposure was classified as having experienced passive exposure to environmental tobacco smoke (maternal urinary cotinine concentration 118.9 ng/ml), while a second neonate was classified as having a mother who was an active smoker (maternal urinary cotinine concentration 338.2 ng/ml). Two neonates whose mothers had declared passive smoking were classified as being active smokers as indicated by the maternal urinary cotinine concentrations of 347.7 and 429.3 ng/ml.

The characteristics of the study population are given in Table 1.

The groups of neonates were comparable in terms of numbers and sex. The difference in respect to method of delivery, maturity, and mean birth weight between the groups was statistically significant. The most frequent method of delivery in non-smoking and passively smoking mothers was cesarean section. The statistically significant dominance of delivery by cesarean section is related to organizational reasons (the questionnaires were more frequently applied to this group of women).

Premature births and lower birth weights were more frequent in neonates born to mothers who were active smokers.

The cytograms of the neonates born to actively and passively smoking mothers was similar with regard to the quality of cells present. Squamous, columnar, and neutrophil cells predominated (Table 2). Analysis of the cytograms of the neonatal nasal mucosa in terms of dominant cells greater than 50% revealed no statistically significant difference between the study groups (Table 3). No correlation was observed between the

Tab. 1. Characteristics of neonatal study groups.

	Neonates of non-smoking mothers (28)	Neonates of passively smoking mothers (29)	Neonates of actively smoking mothers (29)	p-value
Sex				0.13
Female	12 (42.9%)	17 (58.6%)	20 (69.0%)	
Male	16 (57.1%)	12 (41.4%)	9 (31.0%)	
Gestational age	36–41 weeks	32–41 weeks	23–41 weeks	0.01
≥37 weeks	26 (92.9%)	25 (86.2%)	16 (55.2%)	
<37 weeks	2 (7.1%)	4 (13.8%)	13 (44.8%)	
Birth weight (g)	1750–3790 3118±456	2070–4470 3484±544	520–4150 2521±1185	0.00
Method of delivery				0.02
spontaneous labor	7 (25.0%)	7 (24.1%)	15 (51.7%)	
cesarean section	21 (75.0%)	22 (75.9%)	13 (44.8%)	
vacuum extractor	0 (0.0%)	0 (0.0%)	1 (3.5%)	
Cotinine concentration (ng/ml) median	< 32.5	32.5–200 62.0	32.5–2091.8 261.34	

Statistical significance $p < 0.05$

concentration of cotinine in maternal urine and the different cytogram profiles (Table 4).

DISCUSSION

The cytological picture of the nasal mucosa in healthy individuals is dominated by columnar cells, mostly ciliated, as well as by goblet cells (in a ratio of 5:1). Single cells such as neutrophils, squamous, and basal cells which have infiltrated the mucosa are also present. A dominance of one of these cell types in the cytogram signifies an irregularity. Eosinophils, when present in the mucous membranes of the nose or lower respiratory tract, always indicate a pathology (Tarchalska-Kryńska *et al.*2000).

In this study, a statistically significant dominance by any of the specific cell types in the cytograms of the neonates born to mothers who were active or passive smokers was not observed. Columnar, squamous, and neutrophil cells were predominant in the groups of neonates born to mothers who were active smokers, passive smokers and non-smokers. No statistically significant association was observed between maternal smoking status and the presence of a given cytogram profile (dominance of one type of cells greater than 50%). Columnar and squamous cell cytogram profiles occurred most frequently and the proportion of the dif-

ferent cytogram profiles was similar in all three groups of neonates.

The normal appearance of cytological specimens of the nasal mucosa of neonates is always characterised by columnar cells (Tarchalska-Kryńska *et al.*2000; Tarchalska-Kryńska *et al.*2002; Tarchalska-Kryńska *et al.*2005). A large number of squamous epithelial cells in the cytogram of the nasal mucosa of the neonate does not indicate the presence of pathology. These cells arise from the nasal vestibule, which at this age, is larger than it is in adults or older children (Tarchalska-Kryńska *et al.*2000). It is not squamous metaplasia of nasal epithelium associated with chronic inhalation of cigarette smoke (Dye & Adler 1994).

This study draws attention to the large number of neutrophils in the cytological smears of the nasal mucosa of the neonates, which does not appear to be associated with the smoking of cigarettes by the mother during pregnancy. Similar findings were observed in other studies (Tarchalska-Kryńska *et al.*2000; Tarchalska-Kryńska *et al.*2002; Tarchalska-Kryńska *et al.*2005). Cohen *et al.* (1985) detected significant neutrophilia in cytological samples taken from the nasal mucosa in 23% of the neonates. Mygind & Winther (1979) refer to this state as one of physiological inflammation, interpreting the occurrence of neutrophils as one of the responses to the presence of bacterial flora

Tab. 2. Relationship between the cytogram distribution of cells and maternal smoker status.

Cells	Neonates of non-smoking mothers (28)			Neonates of passively smoking mothers (29)			Neonates of actively smoking mothers (29)			p-value
	Median	25%	75%	Median	25%	75%	Median	25%	75%	
Neutrophils	14.0	0.0	38.7	5.8	0.0	45.2	12.0	0.0	95.7	0.5
Eosinophils	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.75
Columnar	24.9	0.0	61.8	29.7	3.4	58.3	38.4	0.0	99.1	0.6
Goblet	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.37
Basal	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0
Squamous	22.6	1.85	76.9	12.9	0.0	59.6	18.8	0.0	91.8	0.44

Statistical significance $p < 0.05$

Tab. 3. Nasal mucosa cytology profile according to maternal smoking status.

Cytogram profile	Neonates of non-smoking mothers (28)		Neonates of passively smoking mothers (29)		Neonates of actively smoking mothers (29)	
	No	%	No	%	No	%
Neutrophil	6	21.4	6	20.7	3	10.3
Squamous	8	28.6	9	31.3	10	34.5
Mixed	4	14.3	2	6.9	6	20.7
Cylindrical	10	35.7	11	37.9	9	31.0
Basal	0	0.0	1	3.5	1	3.5

or irritant factors. The current study, as well as its forerunner (Tarchalska-Kryńska *et al.* 2000; Tarchalska-Kryńska *et al.* 2002; Tarchalska-Kryńska *et al.* 2005), confirmed the absence of goblet cells in the nasal mucosa smears of the neonate sampled in the first few days after birth. Goblet cells are secretory cells which produce a secretion containing immunoglobulin S-IgA, which commences between 6–8 weeks of life and allows the elimination of infectious microorganisms and allergens from the nasal mucous membrane (Holt 1994).

In line with the study by Vinke *et al.* (1999), who performed biopsies of the nasal concha in children who spent time in the company of persons who smoked more than 15 cigarettes daily, and who had detected an increase in eosinophils in the mucosal membrane of the nasal concha, we looked for similar changes in the study of neonates.

In our study, eosinophils were not detected in the children born to mothers who were active or passive smokers alike. Nor were they detected in the neonates born to non-smoking mothers. No increase in eosinophilia was detected by Cohen *et al.* (1985) in a study assessing the cytology of nasal mucosa of children from the second day of life up to the end of the first year of life.

The toxicity of tobacco smoke on fetal development is primarily associated with the damaging effect of nicotine, the appearance of abnormal forms of hemoglobin, chiefly carboxyhemoglobin, as well as with morphological and functional changes of the placenta (Lambers & Clark, 1996; Pastrakuljic *et al.* 1999). It leads to chronic hypoxia and undernutrition manifested in the neonatal period mainly by an increased incidence of prematurity (Shah & Bracken 2000) and intrauterine growth restriction (Pastrakuljic *et al.* 1999). Restriction of intrauterine growth affects all of the morphological features (body mass, weight, head and chest circumference (Pastrakuljic *et al.* 1999), and also the internal organs such as the brain, lungs, and kidneys (Anblagan *et al.* 2013).

The amniotic fluid of mothers who are very active smokers during pregnancy is a decidedly hostile environment for the developing fetus, because nicotine passes directly to the fetus (Hakkola *et al.* 1998; Pastrakuljic *et al.* 1999; Benowitz *et al.* 2009). Concentrations of nicotine in fetal serum and amniotic fluid are slightly higher than in maternal serum (Benowitz *et al.* 2009). The metabolism of nicotine during fetal life is slower, similar to that of neonates in the first week of life, owing to low CYP2A6 activity and low hepatic blood flow immediately after delivery due to the patency of ductus venosus (Dempsey *et al.* 2000; Gow *et al.* 2001). Cotinine, a metabolite of nicotine, is the best biomarker of tobacco exposure (Benowitz *et al.* 2009). Therefore we have used cotinine to assign maternal smoking status.

In our study there was no correlation between the concentration of cotinine in maternal urine and the cytogram profile of the nasal mucosa of neonates born to actively and passively smoking mothers. Similarly,

Tab. 4. Correlation between cotinine concentration in maternal urine and nasal mucosa cytogram profile.

Cytogram profile	Cotinine concentration r – correlation coefficient	p-value
Neutrophil	r=0.004	0.97
Eosinophil	r=0.129	0.24
Cylindrical	r=0.083	0.44
Goblet	r=0.179	0.1
Basal	r=-0.065	0.55
Squamous	r=-0.086	0.43

Statistical significance $p < 0.05$

Hermans *et al.* (2001), has not shown an association between maternal smoking during pregnancy and alterations of the development and secretory function of the pulmonary epithelium of the distal airways and the alveoli in infants born at term.

During intrauterine life the respiratory system does not perform its function. Gas exchange takes place through the placenta. The role of the nose which consists of warming, humidifying, and cleansing the air entering the lower respiratory tract is still inactive at that stage. It is therefore not possible to inhale the harmful constituents of tobacco smoke with their local effect on the epithelium of the nasal cavity. Although nicotine (one of the most toxic components of tobacco smoke for the fetus) and its metabolites reach high concentrations in amniotic fluid 128 ng/ml (Jauniaux *et al.* 1999) and 142–162 ng/ml (Rühle *et al.* 1995), in our study they do not have a negative effect on the structure of the nasal mucous membrane of the studied children. The fetal period is probably too short and exposure to other irritating substances of tobacco smoke is too low (placental barrier) to cause changes in the nasal epithelium of neonates.

In conclusion, our study shows that active and passive smoking by women during pregnancy does not have an effect on the cytological appearance of the neonatal nasal mucosa. The association between smoking during pregnancy and the appearance of asthma in early childhood, as well as the dysfunction of the respiratory system, should be more likely linked with the general systemic effect of tobacco smoke on the developing fetus, its chronic malnourishment and hypoxia, an altered fetal immune function, as well as the exposure of the small child to tobacco in the home environment.

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Conflict of interest

None of the authors declared any conflict of interest.

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