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Article

Influence of Selected Lifestyle Factors on Nonylphenol Concentration in Breast Milk of Mothers in Slovakia

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Abstract: Nonylphenol is a endocrine disruptors who belongs to alkylphenol groups. It's high occurrence in the environment can caused an adverse effect on human health. The breast milk can serve as a marker to take measure of human exposure to these chemicals through different routes of exposure. In this study, the influence of different factors (including the length of accommodation, kind of wastes mothers drink, con summation of fish, pork and beef; using gloves, mail polish, using vitamins and medication) on the concentration on nonylphenol in breast milk were studied. The length of accommodation has a statistically significant effect on nonylphenol, concentration in breast milk (p value was 0.02). Other mentioned factors had not a significant influence on nonylphenol values.

Keywords: nonylphenol; breast milk; HPLC-SPE analysis; breastfeeding; lifestyle

1. Introduction

Alkylphenols belong to a group of endocrine disruptors (ED) and xenoestrogen, which are exogenous substances disrupting the endocrine system. They can disrupt the hormonal structure of the body and have adverse effects on human health. The growing attention regarding the health of the next generation is devoted to the effect of endocrine disrupting chemicals on human health [1–4]. Nonylphenols are used for the production of nonylphenol polyethoxylates (NPEOs), the most often used nonionic surfactants in household products, personal care products, detergents, emulsifiers, additives in cosmetic products, water-based paints, textiles, in production of plastics, resins, paints, cleaners and pesticides etc. [5]. Nonylphenol is a mixture of different isomers (ortho-, meta- or para-), which are located by the arrangement of the carbon chain, whereas the most common isomer is the para isomer (4-NP). 4-NP is a derivative of phenol, where a non-alkyl carbon chain with nine carbon atoms is attached to its aromatic ring. It is a potential threat to the environment because it is toxic to aquatic organisms and can have harmful effects on the ecosystems of waterways and seas [6]. In some legislation, measures have been taken to limit the use of NP and its compounds in some products and industrial processes in order to minimize the risks associated with this substance.

NP and NPEOs are regulated in the European Union under Directive 2003/53/EC, which was adopted on 18 June 2003 and became effective on 17 January 2005 [7]. This directive sets restrictions

on the use of these substances in various products. According to this directive, it is prohibited to market and use textile products that contain NP and NPEOs in concentrations higher than 0.1 percent by weight. In addition, the use of these substances in washing preparations, detergents and cleaning agents is prohibited if it leads to the emulsification of hazardous substances in water. However, it is possible that the rules and regulations can be changed and supplemented. Therefore, it is important to follow current information and updates from the European Union and important countries regarding the use of NP [8–10].

Recently, the high occurrence of persistent endocrine disruptors in environment can caused growing concern about their impact on human health. Infants are particularly sensitive to hormonal influences of environmental chemicals. Due to their presence in human milk, concerns for mother's and children's health are increasing [11,12]. Since the lipophilic character of ED can be accumulated in maternal body fat, can pass through the placental barrier along with residual contaminants and can be transferred from plasma into breast milk during lactation period [5,13].

Breast milk is among the body fluids most suitable for monitoring the effect of exposure to alkylphenols to both mother and child [14]. Breast milk is a complex and variable fluid that evolved to nourish the infant and protect it from disease while its immune system continues to develop. The composition of human breast milk varies depending on many factors that correspond to the needs of the child according to its age and other factors [15–17]. It is an irreplaceable form of nutrition for newborns and it's consumption provides important benefits for the newborn immune system, growth, psychological development. Breastfed babies have a more stable and less diverse intestinal microflora than formula-fed babies, but they have more than twice the number of bacterial cells. This may be partly due to changes at the level of the intestinal mucosa due to bioactive substances in human milk. Moreover, breastfeeding reduced the risk of developing different allergies, infections and other chronic diseases, decreased mortality and morbidity in infants as well as occurrence of cancer and osteoporosis in mothers [18]. Therefore, breastfeeding, which is the only source of nutrition for infants and babies can be the route for contamination of children. The breast milk can serve as a marker to take measure of human exposure to these chemicals through different routes of exposure. The main routes of human exposure is through chemicals residue in food, water and air [6,19–21]. NP is excreted by the organism and therefore its concentration in breast milk can be affected by various factors such as diet, environment and individual differences between people. However, it is important to emphasize that the presence of NP in breast milk varies between regions and women.. It is important to remember that the benefits of breastfeeding for the baby outweigh the potential risks associated with exposure to NP.

Mostly chromatographic methods have been used for the determination of alkylphenols. It can be gas chromatography (GC)(with mass spectrometry, MS) [22–25]. high performance liquid chromatography (HPLC) (with fluorescence detection, FSC) [26,27]. or liquid chromatography with mass spectrometry (LC-MS) [28–33].

In our study, the high-performance liquid chromatography with fluorescence detection was used for determination of 4-nonylphenol in breast milk. Considering the complexity of the breast milk samples and the low concentration levels of nonylphenol ($\mu\text{g/L}$), HPLC analysis of these substances requires the sample pretreatment. The main goal of sample preparation technique is to remove potential interferents (which can affect sensitivity and accuracy of HPLC method) as well as concentration of the analyte. The most frequently used technique for sample treatment is solid phase extraction (SPE) based on affinity of analytes to the sorbent [11,27,34–36]. Different types of SPE sorbents (C18, Oasis HLB, Strata-X etc.) are available for extraction of target analytes. Breast milk samples was firstly extracted with liquid-liquid extraction and the extract was treated using SPE.

In the present study, an HPLC-FSC applying Chromabond C18ec SPE sorbent was used for the determination of 4-nonylphenol in breast milk samples.

2. Materials and Methods

2.1. Mothers' and children's characteristics

In the study were 89 nursing mothers were included, of which 73 mothers lived in the city and 16 in the village. Characteristics of mothers and children are introduced in Tables 1 and 2.

Table 1. Maternal characteristics.

Parameter	Mean \pm STD	Education	Percent (%)
Age	32.29 \pm 3.68	High-school	89.90
Weight [kg]	65.11 \pm 12.02	Secondary school	10.10
Height [cm]	167.01 \pm 5.90	Pregnancy	
		- physiological	91.00
		- risk	9.00
BMI	23.32 \pm 4.02	No of Pregnancies	
		1	57.30
		2	27.00
		3	15.70
First menstruation age	13.18 \pm 1.38	Spontaneous birth	78.70
		Surgical birth	21.30

Table 2. Characteristics of children.

Parameter	Mean \pm STD
Child's age in months	4.59 \pm 7.86
Weight at birth [g]	3506 \pm 525.24
Child's lenght at birth [cm]	50.24 \pm 1.98
Apgar score after birth	9.75 \pm 0.67

2.2. Milk sample collection and storage

Each nursing mother enrolled in the study took a sample of breast milk, in the amount of 15 ml. The nursing mothers were from the University Hospital in Bratislava. Samples were transported on dry ice to the laboratory and subsequently dispensed into micro tubes of 1 ml each. Breast milk was stored at -80°C. The concentrations of nonylphenol in breast milk were determined by HPLC method.

2.3. Reagents and Chemicals

4-nonylphenol and 4-octylphenol were supplied by Sigma Aldrich (Steinheim, Germany). Formic acid (98%), methanol (HPLC gradient grade), acetonitrile (HPLC gradient grade) and water were purchased from Merck (Darmstad, Germany).

The Chromabond C18ec (100 mg/1 ml, 45 μ m) was obtained from Macherey-Nagel (Germany). SPE experiments were performed using a Visiprep™ SPE Vacuum Manifold DL (Sigma Aldrich, Germany). After elution, samples were evaporated under the nitrogen and residues were dissolved in mobile phase.

2.4. Sample pretreatment

Breast milk samples were incubated at 37 °C. 0.5 ml of 0.1% (v/v) formic acid in methanol was added to 0.5 mL milk, sample was vortexed for 3 min and centrifuged at 14,000 for 10 min. To supernatant was added 0.5 ml of methanol and sample was centrifuged for 10 min. Then, to the supernatant (450 μ l) was added 50 μ l of 4-octylphenol as internal standard to final volume of 0.5 ml,

which was further pretreated by solid-phase extraction. Individual steps of SPE procedure are summarized in Table 3.

Table 3. Solid-phase extraction procedure using C18ec sorbent for target analytes.

Extraction steps	Chromabond C18ec
Conditioning	1 ml methanol 1 ml water
Sample load	0,5 ml with ISTD
Cleaning	1 ml 5% methanol
Drying	under vacuum 1-2 min
Elution of analytes	1 ml acetonitrile

2.5. HPLC analysis

All analysis were performed using an HPLC (HP 1200, Agilent, Munich, Germany) chromatograph with fluorescence detection. Chromatographic separation was achieved on a Nucleodur C18ec column (150 x 45.6 mm, 5 µm, Macherey-Nagel, Germany) with a precolumn C18 (10 x 4.6 mm, 5 µm). Mobile phase consisted of acetonitrile (A) and water (B). The linear gradient was following: 0-3.9 min 30% B; 4 min 70% B; 4.2-10 min 100% B; 10.1 min 30% B; 13.5 min stop. Flow rate of mobile phase was 1 ml/min. Sample injected volume was 25 µl.

The quantification of 4-NP was performed using internal calibration curves with octylphenol (OP) as internal standard. Linearity of calibration curves were estimated for concentration range 0.1-100 ng/ml (n=7) of NP and 10 ng/ml for OP. Coefficients of determination (R^2) obtained by regression analysis was higher than 0.99.

2.6. Questionnaire

The study used a self-constructed questionnaire that contained 50 questions. The questions dealt with the education and accommodation of the nursing mother, the health status of the baby and nursing mother, socioeconomic status, use of cosmetics and cleaning products, as well as eating habits.

2.7. Ethics committee and informed consent

The Research Ethics Committee of the Slovak Health University in Bratislava approved the project in accordance with the rules and guidelines for human research before the start of research in 2022 (n.13/2022). Participants were informed about the development of the research and those who agreed to participate signed an informed consent form, in two copies, one of which was given to the nursing mother.

2.8. Statistical analysis

Data were analyzed using SPSS (Statistical Package for the Social Science, Chicago, IL, USA) for Windows, version 19. The final model was tested by structural equation modeling (SEM) was used for statistical. This software provides extensive possibilities for manipulation, analysis and presentation of data. Descriptive statistics were performed - means, medians, variances, standard deviations and percentile values. We also performed inferential statistics - t-tests and analysis of variance (ANOVA).

3. Results

The concentrations of nonylphenol in breast milk can be influenced by various factors, including the place of accommodation of the nursing mothers. Of the variables determined in the questionnaire, the length of accommodation has a statistically significant effect on nonylphenol concentrations in

breast milk. The longer nursing mothers live in a given place of accommodation, thus they have lower concentrations of nonylphenol in breast milk (Table 4).

Table 4. Statistical evaluation of effect of accommodation's length on nonylphenol concentration in nursing mothers.

Nonylphenol Age Weight/kg Height/cm BMI Length of accomodation					
/ Pearson					
Correlation	0.16	0.02	0.01	0.02	- 0.26
Sig.(2-tailed)	0.13	0.87	0.96	0.87	0.02
N	89	89	89	89	86

Nonylphenol (NP) is a chemical used as an additive in some industrial processes and products, and it can enter the environment. Therefore, it is possible that it can also occur in the home environment. Next factor may include environmental pollution. If the nursing mother's accommodation is near industrial zones, waste dump or other sources of pollution, there is a higher probability that NP can get into her body and subsequently into her breast milk. If a nursing mother consumes food or water containing NP, it is possible that this substance can accumulate in her body and pass into her breast milk. But also the use of products containing NP, such as use of cleaning agents, cosmetics or other products, can contribute to higher concentrations of this substance in breast milk. Nursing mothers who live in the village had increased concentrations of nonylphenol, but these findings were not statistically significant. The assumption was that they could live near an industrial zone or in a polluted environment despite the fact that they live in a village.

In our questionnaire, we found out the birth weight, length and Apgar score of the child. The Apgar score is a simple method that is used to evaluate the health status of a newborn in the first minutes of life (Table 3). It is determined concerning five criteria - heart rate, breathing, muscle tone, response to irritation, skin color. This score provides a quick and objective value of the newborn's health status. We found that the measured concentrations of nonylphenol in breast milk do not affect any of the given parameters in the newborn, for example neither the birth weight, nor the birth length, nor the Apgar score of the child. In the questionnaire, we found out what kind of waters mothers drink; whether mothers consume fish, pork and beef. Results are showed in Tables 5 and 6.

Table 5. Statistical evaluation of water drinking in nursing mothers.

Kind of water	N	Mean±STD	Std.error	95% confidence	Minimum	Maximum	
				interval for mean			
				Lower	Upper	bound	
				bound	bound		
Bottled	22	1.18±0.29	0.06	1.05	1.30	0.38	1.69
Tap	53	1.16±0.31	0.04	1.07	1.24	0.67	2.27
Other	14	1.08±0.23	0.06	0.95	1.21	0.57	1.47
Total	89	1.15±0.29	0.03	1.09	1.21	0.38	2.27

p = 0.587

Table 6. Statistical evaluation of meat consumption in nursing mothers.

Consumption of fish	N	Mean±STD	STD error	<i>p</i> = 0.456
			mean	
NP yes	79	1.14±0.30	0.03	
no	9	1.22±0.21	0.07	
Consumption of pork	N	Mean±STD	STD error	<i>p</i> = 0.073
			mean	
NP yes	60	1.11±0.32	0.04	

no	29	1.23±0.21	0.04	
Consumption of beef				
NP yes	48	1.13±0.35	0.05	p = 0.343
no	40	1.18±0.21	0.03	

In Table 7 we can see the influence of gloves using and nail polish in nursing mothers on nonylphenol concentration.

Table 7. Statistical evaluation of use of gloves and nail polish in nursing mothers.

Using gloves for cleaning	N	Mean±STD	STD error mean	
NP yes	19	1.13±0.28	0.06	p = 0.709
no	69	1.16±0.29	0.04	
Using nail polish				
NP yes	18	1.13±0.28	0.07	p = 0.729
no	70	1.15±0.29	0.04	
Gel nails				
NP yes	17	1.21±0.28	0.07	p = 0.06
no	70	1.14±0.29	0.04	

Table 8. Statistical evaluation of maternal vitamins and drug use in nursing mothers.

Vitamins supplements	N	Mean±STD	STD error mean	
NP yes	60	1.18 ± 0.29	0.04	p = 0.06
no	27	1.06 ± 0.29	0.06	
Medication				
NP yes	23	1.24 ± 0.34	0.07	p = 0.093
no	65	1.12 ± 0.27	0.03	

The lowest and highest concentration of NP in breast milk was 0.67 ng/mL and 2.27 ng/mL, respectively. The average concentration ±STD was 1.16±0.27 ng/ml. None of the mentioned factors had a significant influence on nonylphenol values. At the border of statistical significance are: use of vitamins (p=0.06) and medication use (p value=0.09), while those who use them have higher values; consumption of pork (p=0.07), mothers who do not consume, have higher values. Finally, mothers with gel nails have higher values of nonylphenol (p value=0.06).

4. Discussion

Breast milk remains the best source of infant nutrition, but constant surveillance is needed to keep it pure. Breast-feeding offers many advantages to neonates and infants and provides a range of short- and long-term benefits for growth, immunity, cognitive and psychological development, as well as protection against infection, allergies, and other chronic diseases. The composition of human milk varies at different stages of lactation, distinct times of the day, during each feed, and even between breasts; it contains powerful growth- and immune-enhancing factors; and suckling is considered as the best and only source of nutrition necessary for the infant during the first 6 months of life. Chemical contaminants in milk, if the level is high enough or if the infant is sensitive enough, interact at many possible physiological levels. Numerous studies have associated breast-feeding with

potential medical and social benefits, which include decreased mortality and morbidity in infants from infectious and other diseases, influence on brain development, increased resistance to chronic diseases, and decreased incidence of cancer and osteoporosis in the mothers. Some mothers avoid breast-feeding on personal or social grounds; but the reasons can be associated with complications including secretion, breast pain, engorgement, or mastitis [37]. The adverse events associated with drug exposure via lactation occur most often in neonates younger than 2 months and rarely in infants older than 6 months [38].

Maternal exposure to insecticides or to other chemical pollutants is rarely a contraindication to nursing unless the exposure is excessive. Sweden and Germany have systematic breast milk monitoring programs that have tested considerable numbers of women over time; however, most countries have done little monitoring for metal, pesticides, or industrial chemicals in breast milk. In the United States the objectives and goals of a breast milk monitoring program for women are as follows: Information should be obtained on women from diverse geographic regions of the United States and from different socioeconomic and demographic backgrounds. Samples should be collected from both rural and urban locations. Previous studies should be extended by testing for an increased number of environmental chemicals in breast milk (e.g., certain heavy metals as well as other chemicals with significant lipid solubility and long biological elimination half-life). Longitudinal information should be obtained during the course of lactation so that the decrease in concentration of the chemical over time can be assessed. Lactating women should be enrolled in the study on a longitudinal basis, donating samples on a monthly basis and then every 2-3 months if lactation continues. Recruitment of participants may be aided by lactation consultants. Harmonization of sampling and analysis protocols should be promoted to improve the comparability of the results [39].

NP is a family of close relatives organic compounds called alkylphenols. They are used in the production of antioxidants, additives to lubricating oils, laundry and dish detergents, emulsifiers and solubilizers. These compounds are also precursors of commercially important nonionic substances surfactants alkylphenol ethoxylates and NPethoxylates, which are used in cleaning products, paints, pesticides, personal care products and plastics. Considering the prevalence of NP in the environment and its potential roles as an endocrine disruptor and xenoestrogen, due to its ability to act with estrogen-like activity. NPs act as endocrine disruptors similar to xenoestrogens they bind to estrogen receptors and competitively inhibit natural estrogens. NPs have been shown to mimic natural hormone 17b-estradiol and compete with the endogenous hormone to bind to the estrogen receptors ER α and ER. The sidewalk analysis with NP was studied in spermatozoa tubule because NP is known to act on the testis [6].

Xenobiotics in milk, in high levels or when the child is sensitive they interact sufficiently on many possible physiological levels. Huge benefits of breastfeeding offset the risks of most xenobiotics used during breastfeeding. Unfortunately, there is a lack of epidemiological data on the probability of adverse effects in breastfed newborns and infants, despite existing information on xenobiotics in breast milk. The finding of toxic chemicals in breast milk creates important questions for pediatric practice, for the field of public health and the environment health research community. Fat levels in human milk are a good indicator of potential future public health and environmental problems. Exposure varies greatly depending on local conditions chemical substances and the eating habits of the population in the studio. This measure is also relevant to the developmental exposure of unborn children. Although the research yielded information about different types of chemicals found in breast milk and with regard to the toxicological aspects of many of these chemicals, there is little data on infant parameters exposure through breastfeeding, including those with a time-dependent nature. Great concerns were expressed about the presence environmental contaminants such as pesticides, heavy metals and (POPs) persistent organic pollutants in breast milk and their possible effects on the infant health and development. In some cases, mothers with known or suspected high levels of contaminants in breast milk due to acute or chronic exposure recommended to reduce or stop breastfeeding.

Breast milk is the basis of healthy development of an individual. In our study, we dealt with the occurrence of nonylphenol in human milk. 4-nonylphenol is a chemical substance that was used

until 2012 [7–10,40,41]. From this year, nonylphenol was only accessible to industry. Since it is a lipophilic substance, we were interested in whether the given substance binds to breast milk and passes into the child's organism in nursing mothers.

A similar study was conducted in Turkey in 2017, where the authors found that mothers who consumed fresh fish had higher concentrations of nonylphenol. Also, mothers who used cleaning products had significantly higher concentrations of nonylphenol in their breast milk [27]. This fact was not confirmed in our study.

Tolerable Daily Intake (TDI) of 5 kg body weight (bw) for NP was proposed by the Danish Institute of Safety and Toxicology [42]. From obtained NP concentration, it is possible to calculate the daily intake. Using average NP concentration 1.16 ng/ml, the daily intake for Slovak infants (with 5 kg bw) consuming 500 ml of breast milk was calculated as 0.12 µg/kg/d. This value is much lower than those obtained in other studies [8,27,43,44]. A similar value of 0.17 ng/ml was published in Japan study [45]. Nonylphenols are persistent endocrine disruptors that are priority hazardous substances of the European Union Water Framework Directive. Their presence in the environment raises growing concerns about their impact on human health. Recent studies have shown that nonylphenol is ubiquitous in commercially available foods and is also present in human blood. Günter et al. [4] determined 4-nonylphenol in 44 infant formula samples. They found that the distribution of nonylphenol isomers depends on the analyzed food. Although some isomeric groups predominate, different distributions are common. Variations are even found in the same food group. Nonylphenol is a complex mixture of isomers, and the estrogenic potentials of each of these isomers are very different. Consequently, an isomer-specific approach is needed to determine the potential toxicological impact of nonylphenol in foods [46].

NPs, octylphenol (OP), NP monoethoxylate (NP1EO), and two octylphenol ethoxylates (OPEOs) (namely, OP1EO and OP2EO) have been detected in human breast milk of Italian women [11]. NP was the contaminant found at the highest levels (32 ng/mL), about two orders of magnitude higher than OP (0.08 ng/mL), OP1EO (0.07 ng/mL), and OP2EO (0.16 ng/mL). In this study group, a positive correlation between fish consumption and the levels of NP in the milk was observed, in accordance with the evidence that seafood represents one of the most important sources of exposure to this group of contaminants in Italy. On the basis of the concentrations found in the breast milk samples, a maximum NP daily intake of 3.94 mg/kg/day can be calculated, which is close to the TDI of 5 mg/kg b.w.

5. Conclusions

Human milk is one way removing the mother's environmental toxic chemicals burden. Assessment of presence and quantity environmental toxic chemicals in breast milk, provide information on maternal toxic load and serves as a marker of prenatal fetal exposure to this chemical.

Based on the obtained results, we can evaluate that breast milk contains concentrations of nonylphenol in reference values and thus does not affect human health. This confirms that nonylphenol is not present in high doses in the mother's body, and therefore does not cause health problems, and breast milk is a safe food for the child. It is very important that such studies are carried out in order to better understand the potential risks to the health of children and mothers.

Risk management should therefore focus on reduce lifetime exposure, especially during the prenatal period and pregnancy, since toxic chemicals accumulate late before pregnancy and are released during pregnancy and lactation.

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