INVESTIGATION OF ENVIRONMENTAL ESTROGENS IN CLINICAL STATES

Ph.D. thesis

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1. Introduction

The term “environmental estrogen” is currently used to cover both plant-derived estrogens (phytoestrogens) and the anthropogenic estrogens (xenoestrogens). Although representatives of both groups are indeed found in the environment, clear distinction must be made between these groups when assessing the risks they might pose to human and wildlife health. Unlike xenoestrogens, which have only recently entered the environment as a result of human activity, phytoestrogens have been present in the biosphere of Earth throughout the evolution of animal life and must, therefore, be considered an integral part of our natural environment. However, substances with natural origin are neither necessarily harmless nor beneficial.

1.1. Phytoestrogens

Phytoestrogens are plant-derived compounds able to bind to the estrogen receptors and exert estrogenic and/or antiestrogenic effects. The vast majority of the phytoestrogens known today belong to the large group of plant phenolics. The major phytoestrogens consumed in substantial amounts by humans are isoflavonoids and lignans.

The effects of phytoestrogens on wildlife and humans have been extensively studied during the last decades. It seems that continuous moderate exposure to these chemicals via plant food could, in fact, be rather beneficial for humans as phytoestrogens may prevent malignancies [1] and improve cardiovascular health [2]. These effects are known to be mediated in part via nuclear receptors including estrogen receptors α and β and the aryl-hydrocarbon receptor. [3, 4] Antioxidant activities also seem to contribute to the phytoestrogens’ beneficial effects. [5]

Caffeic acid is one of the most common plant phenolics. It is found in several sources, such as chicory coffee, artichoke, olive oil and red wine. It can influence the serum estrogen level, and exerts anti-inflammatory effects, in part due to its antioxidant capability. Caffeic acid and its derivatives have been reported to inhibit platelet aggregation in vitro and in vivo. [6, 7]

1.2. Xenoestrogens

After World War II a vast array of novel synthetic chemicals was manufactured, and many of them have become widespread environmental contaminants. From these compounds those which have been shown to exhibit estrogenic effects are called xenoestrogens, and exhibit high chemical structural diversity.

Many of them are organochlorine substances that are associated with small particles in the air so they can travel long distances to areas far away from where they were released into the environment. They are ubiquitous (distributed worldwide) environmental contaminants and have been detected in air, soil and water. They are resistant to biodegradation: their half lives can extend to decades. Animals and humans are unable to degrade/detoxify and/or excrete these substances, hence they are persistent in the environment and accumulate in the food chain. Owing to their lipophilicity, they bioaccumulate especially in tissues of high fat content. Therefore, the literature classifies them as peristent organic pollutants (POPs).

The xenoestrogens’ mechanism of action includes: a./ direct hormone agonism or antagonism; b./ modulation of endogenous hormone levels; c./ or alteration of hormone

receptors. [8] These mechanisms accomplish the definition of endocrine disruption, hence xenoestrogens are estrogen-like endocrine disruptors. Since xenoestrogens occur as mixtures in the environment, usually more than one compound is involved in exposures.

The majority of the POPs are polyhalogenated aromatic hydrocarbons. Three major groups can be distinguished: polychlorinated biphenys (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). The particular compounds vary by the number and position of chlorine or bromine substituents.

The vast majority, more than 90% of human PCB and PCDD/F exposure comes from dietary sources – especially high fat foods of animal origin –, other sources such as exposure via air or water are negligible. [9] These compounds are known endocrine disruptors, several papers have reported their biological effects in the last decades. They can play a role in the growth deficits of foetuses and infants, and in the impaired psychomotor, neuropsychological and neurocognitive development in infants and in children. [10-12] Moreover, they can affect the function of the reproductive organs, the thyroid gland and the immune system [13, 14], and can contribute to cancer development. [15]

1.3. Macrophage migration inhibitory factor (MIF)

MIF has been the first to be described in 1966 [16] of the immunological mediators called later cytokines. It was initially isolated from the supernatants of activated T-lymphocytes and was found to inhibit the random migration of macrophages. [16] Later a variety of cells have been identified that produce or release MIF. It is a key cytokine in several human diseases: e.g. endotoxemia and exotoxemia [17, 18], delayed-type hypersensitivity, arthritis [19], transplant rejection, tumor growth and angiogenesis [20, 21], inflammatory bowel diseases, acute respiratory distress syndrome, atherosclerosis, and many others.

MIF has been demonstrated to have enzymatic activity, an unusual property that distinguishes this factor from other cytokines. Accordingly, MIF has been termed cytozyme or secreted enzyme. The substrate of its tautomerase activity can be D-dopachrome, L-dopachrome alpha methyl ester as well as phenylpyruvate or p-hydroxy-phenylpyruvate. [22, 23] Certain anti-inflammatory polyphenols including also phytoestrogens inhibit the tautomerase activity in a concentration-dependent manner. [24] Although the exact role of the enzymatic activity of this cytokine remains to be delineated, MIF tautomerase has already attained a reputation as a promising pharmacological target for novel small molecular weight inhibitors in inflammatory conditions.

2. Investigation of certain clinical effects of chicory coffee consumption

2.1. Introduction and aims

The favorable effects of moderate red wine consumption on certain cardiovascular risk factors have been documented. The possible benefits of coffee, the other widely popular polyphenol-rich beverage, still await clarification. The influence of coffee consumption on lifetime cardiovascular risk has been analyzed recently in two large cohorts. A dose-dependent protective effect has been found that is more pronounced in female consumers. [25, 26], but is independent of caffeine. The antithrombotic effect of coffee has also been reported to be independent of its caffeine content, but rather attributable to its phenolic compounds being able to be incorporated into the platelet membranes. [27]

A cup of coffee is an abundant source of chlorogenic acid, i.e. caffeic acid esterified with quinic acid. Caffeic acid becomes available for absorption following hydrolysis of chlorogenic acid in the gut. Known to be absorbed almost totally (~95%) from the small intestine caffeic acid reaches its peak blood level within 2 h after oral intake. [28] Chicory (*Cichorium intybus*) is one of the richest dietary sources of caffeic acid and its derivatives, e.g. chlorogenic acid. Coffee brewed from ground roasted chicory roots does not contain caffeine and has a long history of being used as a coffee substitute or admixture.

The proinflamamtory MIF cytokine is attributed to have importance in several inflammatory conditions. Certain antiinflammatory phytochemicals inhibit the tautomerase activity of MIF in a concentration-dependent manner. [24] Among these molecules caffeic acid exhibits one of the best inhibitory potentials concerning either phenylpyruvate- or dopachrome- tautomerase activities. [24, 29] Furthermore, caffeic acid and its derivatives have been reported to inhibit platelet aggregation in vitro and in vivo [6, 7] – an effect confirmed in our laboratory as well.

We aimed to investigate the clinical effects of chicory coffee consumption on platelet aggregation, on hemorheological factors and on serum MIF levels.

2.2. Methods

Study participants:

In our self-controlled study 27 healthy volunteers (13 women, 14 men) were recruited. The mean age was 23 ± 0.4 years, the mean body weight was 59.2 ± 2.5 kg for women and 85.5 ± 3.9 kg for men. The volunteers were asked to refrain from consuming alcoholic beverages (e.g. wine), (Arabic) coffee or tea one week before and during the whole period of the study, because these also contain substantial amount of caffeic acid.

Protocol:

The volunteers consumed 3 dl coffee in the morning each day (brewed from 20 g ground chicory granulates) throughout the one week study period. The aim was to observe the effects of both, a single dose and a week long daily consumption of chicory coffee on the following parameters: platelet aggregation, hemorheological factors and MIF concentration. Blood samples were collected at three time points: before the first coffee consumption in fasting conditions, 2 h after the first coffee consumption, and after one week of daily chicory coffee consumption on day 8.

Platelet aggregation:

Platelet aggregation was measured in a Carat TX-4 platelet aggregometer (Carat Diagnosztika Kft., Budapest). Collagen (2 μg/ml), epinephrine (10 μM) or adenosine diphosphate (5 and 10 μM ADP) were used as inductors.

Whole blood and plasma viscosity:

Whole blood and plasma viscosity values were determined with a Hevimet 40 capillary viscometer (Hemorex Kft, Budapest).

Red blood cell deformability:

Red blood cell deformability was determined at various shear stresses by laser diffraction analysis, using an ektacytometer, LORCA (Laser-assisted Optical Rotational Cell Analyzer, RR Mechatronics, Hoorn, Netherland). Elongation indexes were calculated from the diffraction patterns for shear stresses of 0.3–30 Pa: a higher elongation index indicates greater red blood cell deformation.

MIF concentration:

Serum MIF levels were assessed by ELISA method with a Duo set ELISA Development System from R&D Systems, Minneapolis, MN, USA according to the manufacturer's instructions.

Statistical analysis:

Data were analyzed using the SPSS statistical software package. For statistical analysis ANOVA repeated measures and paired t-test was used. A 'p' value <0.05 was considered to be statistically significant.

2.3. Results

Platelet aggregation:

Caffeic acid inhibits platelet aggregation in vitro. [6] This finding has been confirmed by us as well: 1.2 mM caffeic acid decreased collagen-induced platelet aggregation in vitro by 50%.

The dietary intervention produced variable effects on platelet aggregation, depending on the inducer used for the aggregation test. While platelet aggregation induced by 5 μM ADP did not change significantly, the 10 μM ADP induced platelet aggregation increased at 2 h and on day 8 as well. Collagen-induced platelet aggregation decreased from baseline at 2 h, however, epinephrine-induced aggregation increased.

Whole blood and plasma viscosity:

The whole blood viscosity decreased significantly after one week daily chicory coffee consumption. Similarly, a significant decrease in plasma viscosity has been observed after one week of chicory coffee consumption. No changes were detected in basic hematological parameters such as hematocrit or fibrinogen levels, the main determinants of whole blood or plasma viscosity, respectively.

Red blood cell deformability:

After one week of daily chicory coffee consumption significant improvements were seen in red blood cell deformability at all fluid shear stresses lower than 30 Pa.

MIF concentration:

Daily consumption of chicory coffee for one week significantly decreased serum MIF levels.

2.4. Discussion and conclusion

The participants observed no relevant side effects during our study, therefore, chicory coffee consumption appears to be a feasible dietary intervention for intake of plant-derived phenolic compounds. Changes in hemorheological parameters and in platelet aggregation detected in the course of this dietary intervention are unlikely to be attributable to only one single compound, but rather to an array of agents absorbed from chicory coffee.

The inhibitory effect of chicory consumption on platelet aggregation seems to vary with the inducer used. Nevertheless, in vivo antiplatelet activity was found much weaker than expected from the preceding in vitro studies with caffeic acid, one of the most abundant plant phenolics in this beverage.

The consumption of chicory coffee for one week significantly improved hemorheological parameters, such as whole blood and plasma viscosity, and red blood cell deformability. These changes indicate that daily chicory coffee consumption might be preventive against certain microcirculatory pathologies. The decrease in whole blood viscosity might largely be attributable to the increased red blood cell deformability. The modest but significant decrease in plasma viscosity in the face of unchanged fibrinogen levels appears perplexing. Further studies are warranted to clarify the exact mechanisms and to identify the compounds behind these effects of chicory coffee.

Baskurt et al. [30] found significantly decreased red blood cell deformability in septic states at fluid shear stresses less than 5 Pa in humans and rats. Elevated MIF levels are acknowledged indicators of septic state. [17] Potato peel extract rich in caffeic acid has been reported to protect red blood cells against oxidative damage, a known cause of impaired cell deformability. [31] The daily consumption of chicory coffee rich in antiinflammatory phenols (including caffeic acid) for one week substantially decreased the serum MIF levels of healthy volunteers in our study in parallel with the improved red blood cell deformability. To decide whether these phenomena are related or are merely coincidental further studies are needed. Notably MIF upregulates adhesion molecules on endothelial cells [32], hence suppression of MIF levels could be regarded as preventive against monocyte adhesion and emigration, a key element in initiation of the atherosclerotic process.

A report by Broekhuizen et al. [33] – published after our results – has confirmed the effect of poliphenol-rich nutriment on serum MIF levels. Administration of a polyphenol rich grape and apple extract for four weeks to participants with cardiovascular risk factors decreased their plasma MIF levels by 11%.

In conclusion, our study constitutes an encouraging starting point to investigate further the antithrombotic, antiinflammatory and beneficial hemorheologic effects of phenolic compounds found in chicory coffee.

3. Investigation of PCBs and PCDD/Fs in Hungarian breast milk

3.1. Introduction and aims

The extensive use of organohalogenic compounds in industry has released an unprecedented number of manmade substances into the environment. Owing to their lipophilic properties and to their high stability, POPs accumulate in the food chain. These compounds are easily transferred to human breast milk because of its high lipid content. [34] Hence breastfed infants are at risk of being exposed to considerable amounts of POPs at the sensitive stage of organ development and maturation. Several studies indicate that exposure to POPs via breastfeeding may contribute to impaired neurological, immunological and endocrinological development in infants and in children. [10-14]

In the last 30 years organochlorine contaminants in breast milk from several countries have been published. However, there is still insufficient documentation from Hungary. It is well known that POP levels in breast milk reflect the previous long-term exposure of the mother and also give information about the exposure of the breastfed infant. We have aimed to provide reliable data on POP levels in Hungarian breast milk and to compare these levels with the ones reported from other European countries. We have also investigated the potential change in concentrations over the course of breastfeeding period. Furthermore, the infants' breast milk-derived POP exposure has been calculated from the data obtained.

3.2. Methods

Study participants:

34 healthy mothers (mean age: 26.7 ± 4.9 years) were recruited who had delivered healthy, full-term infants at the Hospital of Baranya County at Pécs in 2007 and their infants were exclusively breastfed in the first three months after birth.

Milk sample collection:

50-50 ml breast milk samples were collected during the first three months of lactation at postpartum days 5, 12, and 84 (colostrum, transition milk, mature milk). The mothers were instructed to sample milk into glass containers pre-washed with acetone in the early morning hours, and milk samples were stored at −20°C until analysis.

Sample analysis:

The analytical procedures were adopted from the US EPA Method 1613 with minor modifications.

Extraction:

Fat content of breast milk samples were extracted using an Accelerated Solvent Extractor (Dionex ASE 300; Dionex, Sunnyvale, CA, USA). A 2:1 mixture of diethyl ether and isopropanol was used for the extraction procedure and the extraction program consisted of two cycles at 1500 psi at +120°C. The extracts were evaporated to dryness, then the extracted lipid content was determined gravimetrically.

Clean-up:

The samples were treated with concentrated sulfuric acid (98%) and transferred onto Extrelut columns (Merck, Germany) for clean-up. Elution was performed the day after with n-

hexane. Then the eluates were loaded onto a neutral alumina column activated at 400°C before use in order to separate PCBs from PCDD/Fs. The alumina columns were eluted with carbon tetrachloride (CCl_4) to elute non dioxin-like (NDL) PCBs and mono-ortho dioxin-like (DL) PCBs. Then dichloromethane (CH_2Cl_2) was loaded onto the columns to elute PCDDs, PCDFs and non-ortho DL-PCB congeners.

Instrumental analysis:

PCB congeners are referred according to the International Union of Pure and Applied Chemistry's nomenclature (IUPAC numbers). The following PCB congeners were targeted: 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 170, 180 and 189. Furthermore, seventeen 2,3,7,8-chlorine-substituted PCDD and PCDF congeners were selected for determination.

The quantitative determination of PCBs and PCDD/Fs was performed by high-resolution TRACE GC 2000, Thermo Finnigan gas chromatograph coupled with high-resolution a Mat 95 XP Mass Spectrometer (HRGC/HRMS).

Assessment of infants' POP exposure:

The assessment of infants' POP exposure via breast milk involves both the analysis of contaminant content of breast milk and the estimation of infants' milk consumption. The exclusively breastfed infants' body weight was measured at the 1., 3., 5., 7., 9., 11. and 13. week postpartum for a 24 h period before and after each feeding. Then the daily breast milk intake was calculated.

Statistical analysis:

Data were analyzed using the SPSS statistical software package. For statistical analysis ANOVA repeated measures and Pearson correlation was used. Multiple linear regression analysis was performed to evaluate POP levels in relation to maternal and infants' characteristics. Concentration values below the limit of detection (LOD) were considered to half of such limit. DL-PCB and PCDD/F levels were also expressed in toxic equivalent (TEQ) concentrations using the toxic equivalent factors (TEFs). A 'p' value <0.05 was considered to be statistically significant.

Infants' exposure to POPs via breast milk depends on the volume of breast milk consumed, on the fat content of breast milk and on its' POP concentrations. Congeners possessing TEF, i.e. DL-PCBs, PCDDs and PCDFs were included in the calculation. The exposure assessment was performed using the following stochastic equation:

$$\text{daily POP exposure} = (V \times F \times C) / 100$$

where V= volume of breast milk intake adjusted to body weight in g/kg bw; F= breast milk fat content in g%; C= sum of DL-PCB és PCDD/F concentrations in pg TEQ/g fat.

3.3. Results

Fat content:

There was considerable variation in fat content of samples from different individuals at each time point. Mean fat contents were lower at the beginning of lactation and tended to increase later, however, this change did not reach significance. The fat contents obtained at the 3 time points were not correlated with each other.

PCB concentrations:

Analysis of the PCB congener profiles showed that the predominant congeners were the NDL-PCBs: 153, 138, 180 and 101. The sum of these 4 congeners accounted for about 75% of the total concentration of PCBs at each time point. The most abundant DL-PCBs were PCB-118 (mono-ortho DL-PCB) and PCB-77 (non-ortho DL-PCB). The DL-PCB concentration accounted for about 11% of the total PCBs at each time point.

PCDD/F concentrations:

Among PCDD/Fs the predominant congener was OCDD. The congeners principally contributing to the total TEQ (derived from DL-PCBs and PCDD/Fs) were always 1,2,3,7,8-PeCDD and PCB 126. The sum of TEQs derived from these two congeners accounted for about 40% of the total TEQ at each time point. The contribution of DL-PCBs to total TEQ was lower than PCDD/Fs at each time point, at around 30%.

Determinants of milk POP levels:

The association between the POP concentrations at day 12 and maternal characteristics related to body burden was evaluated by multiple linear regression analysis. Only parity was found to be significantly negatively associated with total NDL-PCBs, total PCBs, as well as total TEQ levels derived from PCDD/Fs and DL-PCBs. The observed values were not associated with maternal age, BMI or rate of body weight increase during pregnancy.

Longitudinal changes of POP levels during lactation period:

PCB levels in each of the 3 samples obtained from individual mothers were in strong correlation when compared to other mothers' levels. The mean values of each PCB congener decreased during the first three months of lactation, although this negative trend was significant only for PCB congeners: 114, 118, 126, 138, 156, 157, 167, 169, 170, 189, and also for total NDL-PCBs, total DL-PCBs and total PCBs. The main decrease in levels of PCB congeners was observed from day 5 to day 12 with 2% - 34% (on average 13%). Than from day 12 to day 84 these congeners' concentrations changed on average by 0.2%.

PCDD/F levels in each of the 3 samples obtained from individual mothers were in strong correlation when compared to other mothers' levels. Mean values of all of the PCDD/F congeners declined during the investigated lactation period, even though significant reduction with time was observed only in the cases of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HeCDD, 1,2,3,6,7,8-HeCDD, OCDD and OCDF. Additionally, analysis of TEQ concentrations showed a significant decrease in total PCDD/Fs-TEQ and in total TEQ derived from DL-PCBs and PCDD/Fs. The elimination of highly chlorinated PCDD/Fs was faster than of the less chlorinated ones. PCDFs were eliminated more rapidly than PCDDs. The highly chlorinated OCDF had at least two times higher elimination rate than other congeners. The main decrease was found between day 5 and day 12, on average 16%, compared to the decrease from day 12 to day 84, on average about 7%.

Assessment of infants' POP exposure:

Infants' body weight increased significantly with advancing age of the infants. It has almost doubled until the end of the third month. A significant parallel rise in the total amount of breast milk consumed per day has been observed. The increase of daily milk intake was attributable essentially to the increase of milk volume consumed per feeding and not to the increased breastfeeding frequency. The changes in body weights and in daily milk intakes were highly correlated, therefore, milk intakes adjusted to body weights were used during exposure assessment. The daily milk intake calculated this way increased to its maximum by the third week, and significantly declined thereafter.

Infants' POP exposure via breast milk was calculated as mentioned above. The daily intakes of PCDD/Fs and DL-PCBs at days 5, 12 and 84 were five- to seven times higher than the tolerable daily intake (TDI: 2 pg-TEQ/kg bw) established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Scientific Committee on Food (SCF). Exposure at day 12 was significantly higher than at the other two days

3.4. Discussion and conclusion

The mean fat content increased during the lactation period which is in accordance with other studies. [35] The standard variations of fat contents were high, although milk samples were always collected in the morning to minimize the variability of fat content. It is known that fat content of breast milk varies not only during the day, but also within the same feeding period and even between the two breasts.

This is the first study that reports POP levels of individual breast milk samples obtained in Hungary. These concentrations are lower than the ones recently measured in European countries. [36-38] There are several potential reasons for this. We targeted young and healthy women who were not potentially at a high risk for exposure to these compounds, e.g. through work-related activities or contaminated foods. The lower levels of PCBs and PCDD/Fs in human milk in our landlocked country might be attributable – at least in part – to the low consumption of marine fish and seafood [39], since these are known as major sources of PCB and PCDD/F exposure. [40]

There are only a few previous reports available concerning POP concentrations in Hungarian breast milk. The country participated in all four rounds of WHO-coordinated international exposure studies of PCDD/Fs and PCBs in human milk [41-44], where pooled breast milk samples were analyzed. Hungary was always ranked as one of the least contaminated European countries. Our results of individual samples are in good agreement with the levels observed in those previous studies. The observed low concentrations might explain why in our study POP concentrations were correlated with parity in several cases, but not with the age of the mother. Although, it has been reported that parity or rather the duration of previous breastfeeding decreases, while the age of the mother increases the concentrations of PCB and PCDD/F congeners in breast milk. [45]

In the present study a declining trend of concentrations of several PCB congeners was observed in breast milk during the first three months of lactation. The decline was the steepest (on average 13%) between days 5 and 12, and much less (on average 0.2%) between days 12 and 84. Our results are in good agreement with previous results that have shown a stronger decrease in milk PCB content during the first month of lactation and found more stable values after two months. [35, 46] Other reports observed small – a few percentages per months – but consistent depuration rates over the course of lactation. [47, 48]

A similar degree of declining trend was observed in our study for the PCDD/F concentrations, with the major decrease between days 5 and 12, and a further persistent decline between days 12 and 84. We have found a 19% decrease of total PCDD/Fs-TEQ during the first three months of lactation. Highly chlorinated PCDD/Fs eliminated faster than lesser chlorinated congeners. PCDFs eliminated more rapidly than PCDDs. These results are fully in accordance with other reports which found a decline of 25% or 18% in total PCDD/Fs-TEQ during the first three months of lactation. [49, 50]

Infants' body weight and daily intake of breast milk increased significantly with advancing age of the infants, and these values also correlated with each other. It is known from previous reports that g/day or in ml/day milk consumption varies considerably from infant to infant due to this correlation. The preferred unit of dose in risk assessment is the

amount of compound consumed per kg body weight. Dividing mean milk consumption by mean body weight can lead to misinterpretation of the dose estimate. Accuracy of the estimated dose is increased by using individual body weight and consumption data taken from the same infant on the same day. Arcus-Arth et al. [51] constructed a dataset by reviewing previous studies that provided data on individual infants' daily milk intake adjusted to their body weight. They found a decrease over time with a highest daily milk intake within the first month. However, that was not a longitudinal examination, data were obtained from nonidentical infants at different time points. In our longitudinal study the daily intake of breast milk per kg body weight was the highest at the end of the first month, and then it declined thereafter.

Albeit the POP levels in Hungarian breast milk samples were at the lower concentration range when compared to data from Europe, at all three time points each infants' daily intake of PCDD/Fs and DL-PCBs via breastfeeding exceeded the tolerable daily intake of 2 pg TEQ/kg bw per day established as tolerable daily intake (TDI) by JECFA and SCF.

In conclusion, we detected consistent presence of toxic POP compounds in Hungarian breast milk. More attention should be taken to infants' exposure to POPs, since at this vulnerable time infants can be easily exposed to large amount of pollutants via breastfeeding.

4. Investigation of MIF in human milk

4.1. Introduction and aims

Human breast milk is a complex biological fluid that offers optimal nutrition for suckling infants. Its immunological, developmental, psychological, economic, and practical benefits are widely acknowledged; therefore WHO recommends exclusive breastfeeding during the first six months of life.

A host of bioactive components have been identified in human milk including hormones, growth factors, and mediators of the innate and acquired immune defense. [52, 53] Several proinflammatory and antiinflammatory cytokines have been detected in human milk at quantities that are possibly of physiological relevance, and the list is still growing. [53, 54] These agents may confer protection complementing the infants' immature immune system and might also modulate the maturation process thereof.

MIF is a proinflammatory cytokine that has been shown to be produced and released by a variety of human cells, and it has been reported to be present in human breast milk as well. [55] The time course of breast milk MIF levels have not yet been analyzed, although it might have relevance concerning the development of the infant's immunocompetence. Therefore we aimed to investigate the course of human milk MIF levels during lactation.

4.2. Methods

Study participants:

21 healthy lactating mothers (mean age: 27.5 ± 4.4 years) were recruited who had delivered healthy, full-term infants at the Hospital of Baranya County at Pécs in 2007.

Protocol:

25-30 ml breast milk samples were collected during the first three months of lactation at postpartum days 5, 12, and 84 (colostrum, transition milk, mature milk). To eliminate diurnal variability mothers were instructed to collect milk samples in the early morning hours. Milk samples were stored at −20°C until analysis.

Measurement of MIF concentration:

Milk samples were centrifuged (13,000rpm, 5min) to obtain the aqueous phase, the floating lipid layer, and the cell sediment. Thereafter the protein concentrations in the aqueous phase were determined by Bradford's method, and MIF levels were quantified using ELISA method.

Statistical analysis:

Data were analyzed using the SPSS statistical software package. Depending on the parameter, ANOVA repeated measures, Pearson correlation, Friedman's test, or Spearman correlation was used, as appropriate. Multiple linear regression analysis was performed to evaluate MIF levels and protein contents in relation to maternal and infants' characteristics. A 'p' value <0.05 was considered to be statistically significant.

4.3. Results

Macrophage migration inhibitory factor was detected in all samples. The time courses of milk MIF levels were found to be similar for each mother throughout lactation. MIF levels obtained from the same individual at different time points significantly correlated with each other.

There was a significant declining trend in MIF levels during the first three months of lactation. MIF levels in breast milk collected at day 84 were significantly lower than in milk samples collected at day 5 or day 12.

Protein contents declined gradually in the aqueous phase of milk samples. Protein contents obtained from the same individual at different time points correlated with each other. The decrease was consistent: protein contents of breast milk collected at day 12 and at day 84 were significantly lower than those of samples collected at day 5, and protein contents of samples collected at day 84 were significantly lower than those of milk samples collected at day 12.

No significant correlation was found between MIF concentrations and total protein levels of milk aqueous phase. Migration inhibitory factor levels expressed as ng/mg total protein did not show any significant change throughout the three months of lactation.

No relationship was found between milk MIF levels and maternal and infants' parameters (age, BMI before pregnancy, BMI before delivery, gestational age at birth, mode of delivery , gender, parity, birth weight). Similarly, these parameters had no effect on total protein concentrations.

4.4. Discussion and conclusion

Breast milk is a rich supply of immunologically active agents for suckling infants, who have an immature and developing immune system which renders them susceptible to infections. However, most of the cytokines that are known to be deficient in the neonate have been found in significant amounts in breast milk. [54] Orally administered cytokines presumably act on the mucosal and lymphoid cells of the oropharyngeal and gut-associated lymphoid tissue and promote the maturation of the immune system. [56]

We found relevant concentrations of MIF in the aqueous phase of human milk collected within the first three months of lactation. Indeed, the concentrations of MIF were about one magnitude higher than those of other cytokines in milk as assessed by their immunoreactivity. [57] MIF concentration is ten times higher in breast milk than in serum [58], that can be the result of an active secretion.

MIF concentrations measured by us are in good agreement with levels known from the literature. In particular, Magi et al. [55] reported first the presence of MIF in human milk obtained at postpartum day 5.

We found consistently declining protein contents of the aqueous phase of the milk at the three time points studied. This is in good agreement with the declining trend of whole milk protein content reported earlier, especially during the first month of lactation. [59, 60]

Several reports attest declining trends in concentrations of immunologically active agents of human milk during lactation. [59] MIF concentrations in the aqueous phase of breast milk also decreased consistently with time after delivery.

In conclusion, we have found that MIF is present in high concentrations in breast milk, especially during the first month of lactation. It might have relevance in the complex immunological interaction between the mother and her child, which confer the infant putative protection against infections during this vulnerable period of life.

5. Summary of novel findings

1. In our clinical study the effects of chicory coffee consumption rich in polyphenols were investigated on healthy volunteers.

    - One week chicory coffee consumption produced variable effects on platelet aggregation, depending on the inductor used.
    - One week chicory coffee consumption significantly improved the hemorheological parameters: the plasma and whole blood viscosity, and the red blood cell deformability.
    - One week consumption of chicory coffee high in polyphenols significantly decreased serum MIF levels - an effect demonstrated first by us, and confirmed by other authors shortly thereafter.

2. In our clinical study the presence of PCB and PCDD/F congeners and their change over the course of lactation were investigated in Hungarian breast milk.

    - This is the first study that reports PCB and PCDD/F concentrations of individual breast milk samples obtained in Hungary.
    - The concentration of several PCB and PCDD/F congeners in breast milk decreased significantly during the first three months of lactation. The decrement was more pronounced between days 5 and 12. Our results may contribute to the accurate determination of the elimination rates of POP congeners.
    - The concentrations of POPs in Hungarian breast milk samples were at the lower concentration range in comparison with data from other parts of Europe.
    - The infants' daily intake of breast milk adjusted to their body weights was the highest at the end of the first month. In our longitudinal study both, the infants' body weight and their daily milk intake was obtained periodically individually over the three months. Hence our study serves exact data for more accurate exposure assessments than the ones attempted before.
    - During the first three months of lactation the infants' daily intake of POPs via breastfeeding exceeded the tolerable daily intake established by JECFA and SCF.

3. In our clinical study the presence of MIF cytokine and its change over the course of lactation were established in breast milk.

    - To our knowledge we have demonstrated first that the MIF levels significantly decreased during the first three months of lactation in the aqueous phase of human milk.

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8. List of publications

Publications in peer-reviewed journals:

1. **Vigh É**, Molnár V, Garai J, Varga T, Koppán M, Bódis J. Endometriózis: az ektópiásan túlélő szövet ártalma. I. rész: Az endometriózis patomechanizmusa. Magyar Nőorvosok Lapja 2009, 72(2): 79-97.

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