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Assessment of estrogenic activity and total lipids in maternal biological samples (serum and breast milk)☆

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ABSTRACT

The present study investigated estrogenic activity and total lipid levels in maternal serum and breast milk. The study was performed with 50 mothers from Fang district of northern Thailand. Maternal serum was collected 5 times, including the second trimester, pre-delivery period, delivery period, and lactating period at day 30 and day 60. Breast milk was collected 7 times, including day 1, 7, 14, 21, 30, 45, and 60 of lactation. There were the same patterns of variation between estrogenicity and total lipid levels both in serum and breast milk. The correlation between serum estrogenicity and serum total lipids was found with a correlation coefficient (r) ranging from 0.403 to 0.661. However, no correlation was found between milk estrogenicity and milk total lipids. The results therefore suggest that lipid contents might be the major factors affecting the variation of estrogenicity levels, and xenoestrogens, which the mother subjects exposed, were lipophilic pollutants. The remarkable findings were that the mean levels of estrogenicity in breast milk were approximately 8–13.5 times higher than those in maternal serum compared at the same period. However, no correlation was found between estrogenicity levels in serum and breast milk, leading to decreased accuracy in predicted infant exposure by maternal serum.

1. Introduction

During the last decade, major concern has been raised on the possible harmful effects on endocrine disrupting chemicals to humans and wildlife. A number of these chemicals have estrogenic potentials and were thus termed "xenoestrogens". It has been hypothesized that xenoestrogens are etiologic factors in the increased incidence of breast cancer and disorders of male reproductive tract, including cryptorchidism, hypospadia, and testicular cancer (Cabaravdic, 2006; Degen and Bolt, 2000; Darbre, 2006; Daston et al., 1997; Fenichel and Brucker-Davis, 2008; Safe, 2004; Vandelac and Bacon, 1999). These chemicals can be classified into three types, namely synthetic hormones (i.e.17α-ethinylestradiol), natural compounds (i.e. phyto-estrogens), and man-made chemicals (i.e. pesticides, plastics, and industrial chemicals) (Baker, 2001; Borrelli, 2007). Hong et al. (2002) predicted the potential of 58,000 chemicals to bind to the estrogen receptor (ER). They predicted that 6903 chemicals had estrogenic activity through ER signaling. Many xenoestrogens are lipophilic chemicals. Once these chemicals enter the human body, they can enter to the bloodstream after ingestion, inhalation, and dermal contact. Due to their lipophilic properties, these chemicals can accumulate in tissue for long half-life periods, and subsequently cause adverse health effects (Darbre, 2006; Massart et al., 2005).

A variety of in vitro assays have been developed for screening estrogenic activity of chemicals, including receptor binding assays, cell proliferation assays, and receptor gene assays in yeast and mammalian cells (Baker, 2001; Safe et al., 1998; Soto et al., 2006). The receptor binding assays have been developed to assess the ability of substances to bind directly to the hormone receptor. Enzyme-linked receptor assay (ELRA) is a newly developed receptor binding assay for xenoestrogens. This assay employs the principle as competitive immunoassay based on ligand–protein interaction. The advantages of ELRA are easy to perform, very fast, and relatively cheap, making them a good choice for large-scale screening (Kase et al., 2008; Li et al., 2004; Seifert et al., 1999; Seifert, 2004).

Many papers have reported the presence of xenoestrogens in environmental samples, such as surface water, wastewater, and sediment (Kase et al., 2008; Korner et al., 1999; Nelson et al., 2007; Shappell, 2006; Vigano et al., 2008; Zhang and Zhou, 2008).

☆The study was conducted in accordance with national and institutional guidelines for the protection of human subjects. The study was approved by the ethical committee of Research Institute for Health Sciences, Chiang Mai University, Chiang Mai, Thailand.

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By contrast, the presence of xenoestrogens in biological human fluids, such as serum and breast milk has few references (Fernandez et al., 2004; Rasmussen et al., 2003). Besides, very little is known about placental transmission of xenoestrogens.

Fang district of Chiang Mai province is currently considered to be the largest producer of tangerine in Thailand. Most area in the district has extensively grown citrus orchard trees because these trees give high productivity all year round. Pesticides are also extensively used throughout the year, especially during late summer and rainy seasons (between May and October). In addition, most farmers do not wear personal protective equipments (i.e. gloves, mask, suit, and boots) during farm work. Therefore, pregnant women in the area might be exposed to the pesticides from both occupational and environmental contributions, and their infants might be exposed to these chemicals through placenta and breast milk.

Maternal blood as well as breast milk has been used as marker of exposure of humans to environmental pollutants, and endocrine disrupters in particular. The determination of estrogenicity in maternal serum implies the variation levels of contaminants from pregnancy to lactating period, whereas the determination in breast milk indicates the exposure levels to the contaminants by the breast-fed newborn. Since many xenoestrogens are lipid soluble chemicals and may be incorporated into serum and breast milk, total lipid levels must be measured for meaningfully investigating their correlation with estrogenicity levels. Therefore, the aim of the present study was to assess estrogenic activity and total lipid levels in serum and breast milk samples among 50 mothers from Fang district of Chiang Mai province, northern Thailand.

2. Materials and methods

2.1. Ethical approval

The research protocol was approved by the Human Experimentation Committee, Research Institute for Health Sciences, Chiang Mai University, Thailand.

2.2. Study population

Between March 2007 and June 2007, all pregnant women at the second trimester (between the 13th and the 26th week of gestation) living in Fang district of Chiang Mai province from northern Thailand were invited to participate in the study and signed written consents. The volunteers (n=50) who had normal delivery and full term gestation (between the 36th and the 40th week of gestation) were enrolled as study population.

Six mL of maternal blood samples were collected 5 times, including the second trimester; pre-delivery period (within 12 hours before entering labor room); delivery period (cord blood, as being representative for an infant blood), and lactating period at day 30 and day 60. Ten mL of breast milk samples were collected 7 times, including day 1, 7, 14, 21, 30, 45, and 60 of lactation. The samples were stored at -20 °C before quantitative analysis of estrogenic activity and total lipids. The serum and breast milk samples of all volunteers were collected completely in November 2008.

2.3. Preparation of serum and breast milk samples

The removal of all steroid hormones in serum and breast milk samples was carried out using dextran-coated charcoal (DCC, Sigma), according to manufacturer's instruction. In brief, DCC 0.02 g/mL was added to serum or breast milk and mixed gently overnight at 0–5 °C. The DCC from suspension was removed by centrifugation at 2000 resolutions per minute (rpm) for 15 min and the supernatant was taken for analysis. The breast milk samples were further treated by centrifugation (12,000 rpm for 10 min) to remove fat component and cellular debris. The aqueous layer was inactivated by boiling for 5 min to remove heat-sensitive proteins and peptides, and centrifuged once (12,000 rpm for 10 min). The supernatant was used for analysis. Dilution series of the serum samples (from 1:2 to 1:10) and the breast milk samples (from 1:25 to 1:150) were prepared in PBS before analysis.

2.4. ELRA for quantitative analysis of estrogenic activity

ELRA was carried out according to the method of Seifert et al. (1999) with substantial modification. Assays were conducted in polystyrene 96-microwell plates. Plates were coated overnight at 4 °C with 150 μL per well of 17β-estradiol-BSA conjugate (Steraloids, Inc) in carbonate–bicarbonate buffer (pH 9.6). All subsequent reactions were incubated at room temperature. After washing with PBST (8 nM PBS, pH 7.6, and 0.05% Tween 20), the surface of the wells was blocked with 300 μL of 7.5% BSA (Sigma) for 2 h. After another washing step, 100 μL per well of samples or standard solutions and 100 μL per well of human estrogen receptor alpha (hERα) were added, and incubated for 1 h. The hERα was obtained from Invitrogen and diluted in PBS-EDTA (1:50) before analysis. After further washing step, a mouse anti-hERα antibody (Dako Cytomation) was diluted in PBS (1:500) and 200 μL per well was added and incubated for 30 min. Following a washing step, a biotinylated rabbit anti-IgG antibody (Dako Cytomation) was diluted in PBS (1:5000) and 200 μL per well was added and incubated for 1 hour. After washing step, 200 μL of a streptavidin horseradish peroxidaes (Zymed) was diluted in PBS (1:8000) and 200 μL per well was added and incubated for 1 h. The plates were washed again, and 200 μL per well of OPD solution (10 μL of 30% H_2O_2, 1 mL of 1 mg/mL OPD, and 20 mL of citrate buffer, pH 4.0) was added. The color development was stopped after 10–15 min with 50 μL per well of 2 N H_2SO_4. The absorbance was measured at 492 nanometers (nm). Data analysis was performed with Microcal Origin software (Microcal, Northampton, MA).

Intra-batch coefficient variations of estrogenic activity were 3.45% for control serum (n=16) and 13.67% for control milk (n=8). The inter-batch coefficeint variations were 14.06% for control serum (n=16) and 15.67% for control milk (n=11). The mean recoveries of estrogenic activity were 112.9% for control serum (n=15) and 107.7% for control milk (n=12). The detection limit was approximately 0.5 μg/L.

2.5. Analysis of total lipids in serum and breast milk samples

Serum triglycerides and total cholesterol were determined using an enzymatic method (Synchron, CX systems, Bekman, Germany). The following conversion formula was used for calculation of total lipids in serum samples (Phillips et al., 1989):

$$\text{Total lipid levels in serum(mg/dL)} = 2.27(\text{total cholesterol}) + \text{triglycerides} + 0.623$$

Milk total lipids were determined by measuring creamatocrit (Lucas et al., 1978). The following conversion formula was used for calculation of total lipids in breast milk samples (Jackson et al., 1988):

$$\text{Total lipid levels in breast milk(g/L)} = 6.24 \times \text{creamatocrit}(\%) - 3.08$$

2.6. Statistical analysis

All statistical analyses were performed using SPSS version 11.5. Descriptive data, including arithmetic mean (AR), standard deviation (SD), 95% confidence interval (95% CI), were analysed. Because of non-normal distribution of all variables, the variables were transformed to a natural log scale to satisfy normality criteria before parametric test.

Pearson correlation and student's t-test were used to investigate the correlation between estrogenicity levels in each period for serum and breast milk samples. The Pearson correlation was also used to investigate the correlation between the levels of estrogenicity and total lipids in serum and breast milk samples. P value <0.05 was estimated as being statistically significant.

3. Results

3.1. General characteristics of mothers and their infants

Arithmetic mean age of 50 mothers was 26 ± 5 years (range 18–34 years) and the mean time of residence in the study area was 14 ± 10 years (range 1–33 years). The mean pre-pregnant and pregnant body mass index (BMI) were 22.3 ± 4.3 kg/m^2 (range 16.4–42 kg/m^2) and 25.1 ± 4.3 kg/m^2 (range 19.2–45.8 kg/m^2), respectively. Twenty-three mothers (46%) were nulliparous, 18 (36%) were primiparous, and 9 (18%) were multiparous. Three mothers (6%) drank alcohol and 1 (2%) smoked during pregnancy. From hospital delivery records, 28 infants (56%) were males and 22 (44%) were females. The mean birth weight, height, and head circumference of infants were 3031 ± 485 g, 49.7 ± 6.7 cm, and 34.7 ± 6.8 cm, respectively.

A positive correlation was found between maternal age and birth weight (r=0.320, P=0.023). Head circumference of infants was positvely correlated with birth weight (r=0.310, P=0.029),