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Ochratoxin A: a naturally occurring mycotoxin found in human milk samples from Norway.

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Abstract

Skaug MA, Størmer FC, Saugstad OD. Ochratoxin A: a naturally occurring mycotoxin found in human milk samples from Norway.

The presence of ochratoxin A (OA) in human milk samples from different regions in Norway has been investigated in order to determine the level of infant exposure to OA from human milk. OA was found in 38 (33%) of 115 human milk samples (range 10-130 ng/l). Two to 26 % of the samples contained more than 40 ng/l OA, which will cause a daily intake of OA from human milk exceeding the suggested tolerable dose of 5 ng/kg bw. Significant regional differences were found.

Human milk, ochratoxin A, mycotoxin

Mycotoxins, such as aflatoxins and ochratoxins are secondary metabolites produced by moulds. Ochratoxin A (OA) is produced by *Aspergillus* and *Penicillium* species growing on cereals and other plant substrates, and is frequently found as a contaminant in animal feed and in human food in temperate areas (1, 2).

Humans are exposed to OA by consumption of food which has been directly contaminated through growth of fungi, and by food products derived from exposed animals. In addition, probably also by inhalation of spores containing mycotoxins (3, 4). Spores from *P. verrucosum*, the major ochratoxin producer in crops in the colder climatic zones, were shown to contain both OA and citrinin (5), another mycotoxin produced by this common mould. Inhalation of spores should consequently be an exposure route taken into consideration.

Seasonal fluctuations in OA levels in cereals are reflected by OA levels in humans and in animals (2). In general the growth of mould, and the production of mycotoxins in the crop depend strongly on environmental conditions such as moisture and temperature, and is generally increased in wet summers.

OA has been shown to be nephrotoxic, carcinogenic, teratogenic and immunosuppressive (6). Acute human intoxication by OA is unlikely to occur at the OA levels generally found in foodstuffs, but the long-term effects of continuous low-dose exposure to OA on human health could be significant. The major target organ of OA toxicity is the kidney. The toxin has been strongly suspected of being involved in endemic Balkan nephropathy, and of being associated with urinary tract tumours in humans (7).

A high incidence of OA contamination of human blood in Europe and Canada suggests a continuous and widespread human exposure to this toxin, although at low levels. Analyses of blood samples from different European countries show OA contamination at concentrations from 100 to 14 400 ng/l (8). The concentration level of OA in human milk is reported to be roughly ≤ 1/10 that in human blood (9).

Different risk assessments of the intake of OA have been performed, and different tolerable daily intakes (TDIs) have been suggested (6, 10, 11). The Nordic Working Group on Food Toxicology and Risk Evaluation has suggested a maximum TDI of OA in humans of 5 ng per kg body weight, based on carcinogenicity studies with OA in rats (12).

In human milk samples highest OA concentrations have been found in Sierra Leone, where thirty-five per cent of the samples contained OA at levels from 200 to 337 000 ng/l OA (detection limit: 200 ng/l) *(*13). In Italy 20 % of milk samples had OA levels in the range of 100 to 12 000 ng/l (14). Lower concentrations were found in samples from Germany (17-30 ng/l in 11% of the samples) (15) and Switzerland (5-14 ng/kg in 10% of the samples) (16). In a Swedish study 23 of 40 (58 %) human milk samples contained OA (range 10-40 ng/l, detection limit 10 ng/l) (9).

Prenatal exposure to OA can occur by transplacental transfer (17). It is reported that the OA concentration in fetal serum is twice the maternal one, indicating a possible active placental transport (16). The health risk of OA to neonates and infants has not yet been evaluated.

The purpose of the present work was to determine the occurrence of OA in human milk samples from different regions in Norway and to determine the level of infant exposure to OA from human milk.

Materials and methods

Reagents

OA was purchased from Sigma (St Louis, MO, USA). A solution of OA (10 μg /ml in methanol) was calibrated spectrophotometrically at 333 nm using the value of 6640 for the extinction coefficient (18). The OA solution was diluted to 10^{-8} M in methanol and stored at -20 °C.

Milk samples

Human milk samples were collected during May-August 1994. One hundred and fifteen milk samples were obtained from women living in three different climatic regions in Norway: Elverum (south-east inland, n=48), Trondheim (middle coast, n=19) and Bodø (north coast, n=48) (Fig. 1). From each mother one sample was taken 3-4 days post partum. No additional personal or dietary information was obtained from the donors. The milk samples were stored in sterile tubes, and immediately frozen at -20 °C until extraction and HPLC analysis.

The study was approved by the regional ethical committee, Norway.

Extraction and clean-up

The method described by Breitholtz-Emanuelsson et al (9) was used with slight modifications concerning the purification and HPLC-conditions and with another confirmation method for OA. A volume of 5 ml methanol, 0.5 ml 1 M HCl, and 5 ml chloroform were added to a 2 ml portion of milk. Instead of a self-packed silica gel

column in a Pasteur pipette, a commercially available silica gel cartridge (Bond Elut Varian) with 500 mg silica was employed for clean-up of the chloroform extract. The silica gel was washed with 5 ml chloroform before the chloroform phase from the extraction was added. The solvent of the purified extract was evaporated to dryness at 40-45 °C under a stream of nitrogen. The residue was immediately dissolved in 300 μl of the HPLC mobile phase by means of ultrasonication. Sample extracts were filtered through a 0.45 μm membrane filter prior to injection onto the HPLC column.

Chromatography

The sample extracts were analysed by HPLC ion-pair technique, at an alkaline pH, and with fluorescence detection. The chromatographic system consisted of a Merck Hitachi L-6200 A Intelligent Pump, fluorescence detector (Merck Hitachi F-1080), interface (Merck Hitachi D-6000), a Rheodyne manual injector, and chromatography software (model Hitachi D-6000 HPLC Manager). Separations were carried out on a Spherisorb S3ODS2 (C-18) column, 4.6 x 150 mm, with 3 μm particles. A 50 μl aliquot of the sample extract was injected onto the column. The mobile phase consisted of 10 mM tetrabutyl ammonium bromide in a methanol-potassium phosphate buffer (pH 7.5) mixture (ratio of methanol to potassium phosphate buffer was 51:49). In order to elute late peaks from the column, the ratio of methanol to potassium buffer was changed to 56: 44 after elution of OA. The determinations were performed at 380 nm (excitation wavelength) and 450 nm (emission wavelength). Flow rate was 0.8 ml/min. OA standard solutions (70 -1750 ng/l) for calibration were prepared every day by dilution of the 10^{-8}M OA stock solution with HPLC mobile phase. For quantitation, peak heights were measured by a Merck Hitachi integrator. The 10^{-8}M OA stock solution was added to milk samples for recovery experiments and for calculation of the detection limit of the whole

procedure. The mean recovery for the whole procedure, calculated from spiked milk samples (concentration range 10 - 500 ng/l), was 75 % (64-84, n= 22). The quantitation limit for spiked milk samples was 10 ng/l OA.

Confirmation

The identity of OA in positive samples was confirmed in two ways:1) As a routine all sample extracts shown to contain OA during initial HPLC analysis were analysed in duplicate, with a direct spiking of the second aliquot. This was performed by adding an amount of a stock solution of OA to the aliquot. The chromatograms of the unspiked and the spiked sample extract were then compared. 2) Derivatization of OA through methylation of the extracts with subsequent HPLC analysis was also used for qualitative confirmation of positive samples (16): A 200 µl aliquot of the purified sample extract was evaporated to dryness, and the residue was dissolved in 2.5 ml methanol and 0.1 ml conc. HCl. The mixture was kept overnight at room temperature. After evaporating the mixture to dryness, the residue was dissolved in 200 µl mobile phase. 50 µl of it was injected onto the HPLC column and analysed (disappearance of the OA peak in the chromatogram and appearance of a new peak with the same retention time as that of the methyl ester).

Statistics

The standard curves used for quantitation were calculated by the least-squares method. The coefficient of variation of the peak height of OA at different injections of the same standard solution was 3 % (within-day variation). The coefficient of variation of the peak height of OA from spiked milk samples was 12 % (between days). Fisher's exact test was used to compare the OA levels in the three areas.
A p value less than 0.05, using a two tailed test, was considered to be statistically significant.

Results

Thirty-eight of the 115 human milk samples (33%) contained OA in concentrations ranging from 10 to 130 ng/l. The incidence and level of OA contamination varied significantly between the three regions (Table 1). Lowest incidence and contamination level was found in the northernmost area, north of The Arctic Circle ($p = 0.0005$, A versus region B+C, see map, Fig.1). In this area only 7 of 48 samples (15%) had detectable levels of OA, and only one sample contained more than 40 ng/l. Fourteen of the 115 samples (12 %) contained more than 40 ng OA /l. In the northernmost area only 1 of 48 samples (2 %) contained more than 40 ng/l, whereas 5 of 19 samples (26 %) from the middle part of Norway exceeded this value ($p = 0.006$).

Discussion

Supposed a 4-kg infant consumes 500 ml of milk per day the milk must contain less than 40 ng OA per l to prevent a daily intake exceeding the suggested TDI of 5 ng/kg bw. The present investigation demonstrates that in Norway 2 - 26 % of the infants were exposed to milk contaminated with OA at levels that exceed the TDI. Significant individual and regional differences in OA levels in the milk were found. In the east and middle part of Norway 42 - 58 % of the human milk samples contained more than 10 ng/l. Our findings suggest that OA contamination may be a potential health problem. Infants are generally more susceptible to exposures to environmental toxins than adults, and may be more vulnerable to their effects. Short-term adverse effects from OA have not been documented in humans at the

concentrations known to be present in human milk. However, because of the continuous intake and the long elimination half-life (35 days in adult humans) (19) the infant may build up high concentrations of the toxin over time. Risk assessments that have been carried out for OA do not differentiate between risk to children and risk to adults. Further investigations are needed regarding the potential long-term health effects of OA intake in the contamination range found in this work.

The prevalence of OA contamination in east- and middle-part of Norway was approximately the same as reported in the Swedish study (9), but the mean values were higher in the Norwegian samples. The prevalence of OA in human milk in Sweden and in Norway is higher than reported from Switzerland (16) and Germany (15). The results may indicate that the average exposure to OA of the population in some regions of Norway is higher than in Sweden, Germany and Switzerland. The geographical variations in the level of OA contamination of the milk seen in this study may be due to differences in dietary habits, and may also be due to regional differences in inhalation exposure to fungal spores. In the northern part of Norway there was a significant lower incidence and level of OA contamination of the milk. There are minor farming activities (grain processing, hay handling etc.) in the northern areas, which might reduce the exposure to airborne fungal spores. To evaluate the regional differences in OA levels in human milk found in this study, more data is needed regarding consumption data and OA levels in foodstuffs in Norway.

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Tab. 1: Occurrence of ochratoxin A in human milk samples from Norway

Detection limit 10 ng/l ochratoxin A

	Concentration of ochratoxin A, ng/l	No of samples		
Region	Mean (Range)	not detected	10-40 ng/l	> 40 ng/l
Bodø (A) n=48	27.4 (10-56)	41	6	1
Trondheim (B) n=19	49.0 (10-102)	8	6	5
Elverum (C) n=48	43.2 (10-130)	28	12	8

Figure 1:

Fig.1 Map of Norway, showing the three sampling regions

A: Bodø, B: Trondheim, C: Elverum