Mycoestrogen Pollution of Italian Infant Food

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Objective To determine the concentrations of zearalenone and its metabolites in the leading brands of infant formula milks and meat-based infant foods commonly marketed in Italy, and to assess their repercussion in the provisional tolerable daily intakes of these estrogenic mycotoxins.

Study design A total of 185 cow's milk-based infant formulas and 44 samples of meat-based infant foods samples were analyzed. The analysis of mycotoxins was performed by immunoaffinity column clean-up and high-pressure liquid chromatography with fluorescence detection.

Results Zearalenone was detected in 17 (9%) milk samples (maximum 0.76 μg/L). The α-zearalenol was detected in 49 (26%) milk samples (maximum 12.91 μg/L). The β-zearalenol was detected in 53 (28%) milk samples (maximum 73.24 μg/L). The α-zearalanol and β-zearalanol were not detected in milk samples. Although α-zearalenol was detected in 12 (27%) meat samples (maximum 30.50 μg/kg), only one meat-based sample was contaminated by α-zearalanol (950 μg/kg). Zearalenone, β-zearalenol, and β-zearalanol were not detected in meat samples.

Conclusions This study shows the presence of mycoestrogens in infant (milk-based and meat-based) food, and this is likely to have great implications for subsequent generations, suggesting the need to perform occurrence surveys in this type of food. *(J Pediatr 2011;159:278-83)*.

Zearalenone is a nonsteroidal mycotoxin produced by *Fusarium sp* on several grains.[1] Despite its low acute toxicity and carcinogenicity,[2] zearalenone exhibits estrogenic and anabolic properties in several animal species, including human beings.[2,3] Zearalenone contamination of food is caused either by direct contamination of grains, fruits, and their products[2] or by "carry-over" of mycotoxins and their metabolites in animal tissues, milk, and eggs after intake of contaminated feedstuff.[3,4] The harmful effects of zearalenone may be increased through its derivatives, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), and β-zearalanol (β-ZAL). Zearalenone and its derivatives have the remarkable ability to mimic estrogen, acting as an estrogen receptor agonist.[5-7] With in vitro assay (MCF-7 human breast cancer cell proliferation assay), the most potent estrogens were α-ZAL and α-ZOL, which had about the same potency as 17 β-estradiol (E_2), and zearalenone was about two orders of magnitude less potent than both of those metabolites.[5,6] The β-ZOL and β-ZAL were between one and five orders of magnitude less potent than zearalenone.[5] The relative potency of zearalenone compared with E_2 in the uterotropic assay was about 0.001, whereas the potency relative to that of E_2 in the vaginal cornification assay was 0.001.[8] The α-ZAL was several times more active in the uterotropic assay than zearalenone.[9]

Infant formula milks constitute an important or often sole source of food for newborns and infants during their first months of life. Recently we have reported the contamination by aflatoxin M1 and ochratoxin A in infant formula milk marketed in Italy.[10] The introduction of complementary food is recommended to begin between the ages of 4 and 6 months for most infants. Nonetheless, only limited information exists about the presence of contaminants in commercial infant food, and none exists regarding zearalenone and their metabolites in meat-based infant foods.[11,12]

The aim of this study was to determine the concentrations of zearalenone and its metabolites in the leading brands of infant formulas marketed in Italy and to assess their repercussion in the provisional tolerable daily intakes of these mycotoxins. Furthermore, we determined levels of zearalenone and its metabolites contamination in meat-based infant food.

α-ZAL	α-Zearalanol
α-ZOL	α-Zearalenol
β-ZAL	β-zearalanol
β-ZOL	β-zearalenol
E_2	17β-estradiol
HPLC	High-pressure liquid chromatography
IAC	Immunoaffinity columns
LOD	Limit of determination
LOQ	Limit of quantification
PMTDI	Provisional maximum tolerable daily intake

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The authors declare no conflicts of interest.

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Methods

A total of 185 samples from 14 brands (5 different batch numbers for each product) of infant formula powders (n = 17) and ready-to-use preparations (n = 20) commonly marketed in Italy were collected from 2007 to 2008 and analyzed. Different types of cow's milk-based formula were classified as preterm formula (n = 11 [1 powder and 10 liquid]) and starter formula for infants from the first day to 6 months of age (n = 26 [16 powder and 10 liquid]). A total of 44 samples from seven brands of meat-based infant foods commonly marketed in Italy were collected in 2008 and analyzed. Different types of meat-based infant food were classified as beef (n = 7), chicken (n = 7), calf (n = 7), turkey (n = 5), rabbit (n = 5), pig (ham) (n = 5), horse (n = 4), and lamb (n = 4).

Zearalenone, α-ZOL, β-ZOL, α-ZAL, and β-ZAL reference standards, acetic acid (analytical reagent grade), and β-glucuronidase from Helix pomatia were purchased from Sigma (Milan, Italy). High-pressure liquid chromatography (HPLC)–grade water, methanol, chloroform, and acetonitrile were supplied by LABSCAN (Hasselt, Belgium). The immunoaffinity columns (IAC) ZearaStar were purchased from Tecna Srl (Trieste, Italy).

Samples Preparation

Milk. Infant formula samples 5 mL were mixed with sodium acetate solution (0.2 M pH 5.5) 4 mL and hydrolyzed for 16 hours at 37 °C with glucuronidase solution 50 μL to cleave the respective conjugates. The sample was then diluted with phosphate-buffered saline solution (pH 7.4) 40 mL. The diluted sample (pH value about 5.5 to 6.0) was filtered through a Whatman filter paper (Millipore Corporation, Maid Stone, United Kingdom). This solution was passed through the IAC at a flow rate of one to two drops s^{-1}. The column was washed with phosphate-buffered saline solution 20 mL (1-2 drops s^{-1}). Elution was performed with methanol 3 mL. The elute was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 250 μL of the HPLC mobile phase. A 100-μL amount of this solution was injected into the HPLC system. For samples of powdered milk, 5 g were suspended in distilled water 30 mL, heated to 50 °C, homogenized, and then processed as for liquid milk.

Meat. Five grams of meat-based infant food were mixed with 2 mL solution 20% acetic acid adjusted to pH 5 by addition of NaOH 1 mol/L and hydrolyzed for 16 hours at 37 °C with glucuronidase solution 50 μL to cleave the respective conjugates. The sample was then mixed with chloroform extraction solution 7.5 mL. Extraction was done by shaking for 20 minutes on a horizontal shaker and spinning in a centrifuge at 3000 rpm for 15 minutes. The extraction step was repeated twice. The resulting organic phases were pooled, mixed with TRIS solution 3 mL (0.04 M pH 7.5), shaken for 10 minutes on a horizontal shaker, and spun in a centrifuge at 3000 rpm for 10 minutes. The organic phase was separated from TRIS solution by use of a Pasteur pipette. The resulting organic phase was evaporated to dryness under a stream of nitrogen. The residue was redissolved in 250 μL of the HPLC mobile phase. A 100-μL amount of this solution was injected into the HPLC system.

Spiked Samples

Samples spiked before extraction were used to check the performance of the extraction and clean-up procedure and to obtain validation parameters. Spiking solutions of mycotoxins were prepared daily by dilution with HPLC mobile phase. For samples of liquid milk, after thoroughly mixing for 30 minutes, the mycotoxins-fortified milks were left for at least 2 hours at room temperature to enable equilibration and used to assay the cleaning procedures before HPLC analysis. For samples of powdered milk, spiked samples were prepared by adding appropriate volume of the spiking solution of mycotoxins to the milk suspension (described above) and then processed as for liquid milk. For samples of meat-based infant foods, after being thoroughly mixed for 30 minutes, the mycotoxins-fortified samples were left for at least 2 hours at room temperature to enable equilibration and used to assay the cleaning procedures before HPLC analysis.

HPLC

The chromatographic system consisted of a Jasco 880 pump and a Jasco 821 fluorescence detector (Jasco, Tokyo, Japan). Jasco Borwin software (Jasco UK, Essex, United Kingdom) was used for data processing. The excitation wavelength (λex) and emission wavelength (λem) were set at 274 and 440 nm, respectively. The reversed-phase column was a Spherisorb Waters C_{18} column (3 μm, 150 × 4.60 mm) connected to a Waters Guard-Pak C_{18} pre-column (4 μm) (Waters, Milford, Massachusetts). The column was kept at room temperature. The HPLC was operated with a mobile phase system consisting of acetonitrile-water (adjusted to pH 4 by addition of H_3PO_4 85%) 55/45% v/v at a flow rate of 1 mL/min.

The HPLC method was validated according to international rules[13]: selectivity, linearity, limits of determination (LOD) and quantification (LOQ), repeatability, and reproducibility were determined. For the linearity test calibration curves with zearalenone and α-ZOL working standard solutions at 1 to 1000 μg/L and with β-ZOL, α-ZAL and β-ZOL working standard solutions at 10 to 1000 μg/L (for each mycotoxin in acetonitrile) were obtained. Milk samples spiked with zearalenone and α-ZOL at 0.05, 0.1, 0.2, 0.5 and 1 μg/L and with β-ZOL, α-ZAL, and β-ZOL at 0.5, 1, 2, 5, and 10 μg/L were analyzed with the IAC clean-up and HPLC method. Taking into account dilution and concentration steps, these spiked samples corresponded to zearalenone and α-ZOL standard concentrations of 1, 2, 4, 10 and 20 μg/L and β-ZOL, α-ZAL and β-ZOL standard concentrations of 10, 20, 40, 100 and 200 μg/L. Meat samples spiked with zearalenone and α-ZOL at 0.1, 0.5, 1, 10 and 50 μg/kg and with β-ZOL, α-ZAL and β-ZOL at 1, 5, 20, 100 and 200 μg/kg were analyzed with the clean-up and HPLC method. Taking into account dilution and concentration steps, these spiked samples corresponded to zearalenone

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