High Growth Rate of Girls with Precocious Puberty Exposed to Estrogenic Mycotoxins

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Objective To test the hypothesis that human puberty timing can be advanced by environmental estrogen exposure.

Study design We analyzed serum mycoestrogen contamination via high-performance liquid chromatography (HPLC) in 32 girls affected by central precocious puberty (CPP) and in 31 healthy female control subjects. All 32 patients received triptorelin (TR) for more than 12 months after diagnosis.

Results Increased serum levels of zearalenone (ZEA; 933.7 ± 200.3 pg/mL; 95% CI, 723.5-1143.9) and of its congener α-zearalenol (106.5 ± 1.9 pg/mL; 95% CI, 104.5-108.5) contaminated 6 girls with CPP, who were from a bounded Tuscany area. At diagnosis, ZEA levels correlated with patient height (r = 0.906, P < .05) and weight (r = 0.887, P < .05), but not with bone age. In patients who were mycotoxin-positive, height (F = 4.192; P < .01), weight (F = 3.915; P < .01), and height velocity (F = 2.777, P < .05) were higher than patients who were mycotoxin-negative during 12-months TR treatment. Height correlated with weight both in patients who were mycotoxin-positive (r = 0.986, P < .001) and in patients who were mycotoxin-negative (r = 0.994, P < .001). Body mass index, bone age, and gonadal secretion was not different in patient groups before and during TR treatment (P > .05).

Conclusions Mycoestrogenic zearalenone is suspected to be a triggering factor for CPP development in girls. Because of its chemical resemblance to some anabolic agents used in animal breeding, ZEA may also represent a growth promoter in exposed patients. (J Pediatr 2008;152:690-5)

Since the 1970s, there has been a worldwide scientific discussion on potential health consequences of human exposure to estrogen disruptors. Many environmentally persistent compounds are toxic estrogen agonists, androgen antagonists, or both. Thus, they can disregulate the hypothalamic-pituitary-gonadal (HPG) axis, potentially inducing central precocious puberty (CPP).1

In humans, little is known about pollutant influence on premature sexual development. In a study of patients with precocious puberty, serum dichloro-diphenyl-dichloro ethylene (DDE) levels were 10-fold higher in foreign girls than in native Belgian girls.2 The authors hypothesized that migration interrupted exposure to estrogen disruptors, inducing accelerated maturation of HPG axis.2 Because native girls without precocious puberty served as control subjects, the association remains speculative. Since it was banned in the late 1960s, DDT and its metabolites are not proposed as major endocrine pollutants in Italy, though DDT/DDE may be detected in biological samples.3

In 1979, Fara et al4 described a school epidemic of premature thelarche in Northern Italy. Italians frequently consumed meat of young animals such as poultry, pig, calf, and lamb, which can be treated with anabolic steroids to increase growth rate. After this episode, the European Union banned the application/use of anabolic growth promoters in agriculture since 1985.5,6

From 1978 to 1984, an epidemic of premature thelarche and precocious puberty occurred in Puerto Rico.7-10 It was suggested that dairy and meat products could be contaminated with anabolic estrogens such as zeranol (α-zearalenol; α-ZAL) or diethylstilbestrol, which were used for increasing muscle mass in cattle and poultry7,8; Schoental suggested the possibility of Fusarium toxin contamination of grain products as
the causative agent. An increased incidence of early thelarche/mastopathy patients in the southeastern Hungary since 1989 was also observed. In that study, estrogenic mycotoxins were detected in 5 of 36 early thelarche patients with serum zearalenone (ZEA) level of 18.9 to 103 µg/L as well. ZEA, α-ZAL, and their metabolites are able to adopt a conformation that sufficiently resembles 17β-estradiol (E2) to allow it to bind to estrogen receptors in target cells exerting estrogenic (agonist) action.

A high incidence of CPP has been reported in a small region of North-West Tuscany: the incidence of CPP in the pediatric population of Viareggio countryside was 22 to 29 times higher compared with that in neighboring areas. Although the cause for this difference remains unexplained, the geographic distribution of CPP strongly suggests the involvement of an environmental estrogen exposure in the onset of CPP.

The aim of this study was to perform serum measurements of mycotoxin ZEA and its metabolites (ie, α-ZAL, β-zearalanol [β-ZAL], α-zearalenol [α-ZOL], and β-zearalenol [β-ZOL]) in North-West Tuscany patients affected by idiopathic CPP and to evaluate the mycosterone exposure as triggering factor for premature sexual development.

METHODS

Subjects

To assess serum mycotoxin contamination, we selected 32 girls with idiopathic CPP who came as outpatients to the Pediatric Endocrine Center of Pisa between 2001 and 2005; 17 girls with CPP were from the Viareggio countryside (group A), and 15 patients were from Pisa (group B). In addition, 31 healthy age- and sex-matched control subjects were selected from Viareggio (n = 15; group C) and Pisa (n = 16; group D). After approval from the local institutional review board, informed consent was obtained from all the parents of patients before the study.

Diagnostic criteria for CPP were consistent with a recent study. At CPP diagnosis, all 32 patients (group A and B) had a history of increased growth velocity, Tanner breast stage of at least 2, and bone age (BA) was advanced >1 year; luteinizing hormone, and follicle-stimulating hormone responses to the gonadotropin-releasing hormone (GnRH) stimulation test (100 mg/m², intravenous bolus dose) were in the pubertal range. Clinical diagnosis of CPP was also confirmed with E2 levels ≥25 pg/mL and by age ≤8 years. After diagnosis, 32 girls with CPP were treated with triptorelin (TR) depot intramuscularly (Decapeptyl, Ipsen Pharma Biotech SA, Toulon Cedex, France) at 0.1 mg/kg body weight every 28 days for >12 months. TR doses were adjusted during treatment according to patient weight. Data on the auxology and the pubertal development were obtained at 3- to 6-month intervals, with BA yearly determined with the Greulich and Pyle method. According to Italian standards, height (Ht), weight (Wt), height velocity (HV), and body mass index (BMI; weight in kilograms/height in m²) are expressed as SD score either for chronological age (SDSCA) and for BA (SDSBa). TR-induced suppression of gonadotropin secretion was checked every 3 or 6 months with radioimmunoassays.

Mycotoxin Measurement

Mycotoxin standards of ZEA, α-ZOL, β-ZOL, α-ZAL, and β-ZAL (Sigma-Aldrich, Milan, Italy) and immunoaffinity columns ZearaStar (Romer Labs, Herzogenburg, Austria) were purchased. Acetonitrile and methanol were of high-performance liquid chromatography (HPLC) grade, and other reagents were of analytical grade (Baker Analyzed Reagent, J.T. Baker, Deventer, The Netherlands).

For mycotoxin analysis, serum samples were collected during the GnRH-stimulating test and at 12 months of GnRHα treatment for CPP, as during both routine evaluations (at baseline and after 12 months) for control subjects. Serum concentrations of mycotoxins were HPLC-assayed. 5 mL plasma was mixed with 5 mL of 0.2 M sodium acetate buffer with a pH level of 5.5. This solution was incubated for 16 hours at 37°C with 50 µL of glucuronidase solution before it was mixed with 15 mL of phosphate buffer saline and adjusting to a pH level of 7.4 with 1 M NaOH. After centrifugation at 3000 rpm, the clear part was passed through ZearaStar column at flow-rate of 1 to 2 drops/s. The column was washed with 20 mL water (1-2 drops/s). Elution was performed with 1.5 mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen. The residue was re-dissolved in 100 µL of HPLC mobile phase and injected into the HPLC system. The chromatographic system consisted of a Jasco 880 pump and Jasco 821 fluorescence detector (Jasco, Tokyo, Japan). Jasco Borwin software was used for data processing. Excitation wave-length (λex) and emission wave-length (λem) were set at 274 and 440 nm, respectively. The reversed-phase column was a Spherisorb 3 μm C18 column (150 × 4.60 mm) from Waters (Milford, Mass). The HPLC was operated with mobile phase system consisting of H2O/ACN (50/50, v/v) at a flow rate of 1 mL/min. For both ZEA and α-ZOL, the limit of detection and limit of quantification were 0.025 ng/mL and 0.05 ng/mL, respectively. For β-ZOL, α-ZAL, and β-ZAL, the limit of detection and limit of quantification were 0.25 ng/mL and 0.5 ng/mL, respectively. The recoveries of ZEA, α-ZOL, β-ZOL, α-ZAL, and β-ZAL were 87.1% ± 0.3%, 84.2% ± 0.2%, and 80.1% ± 0.3%, 79.5% ± 0.5%, and 82.1% ± 1%, respectively. The intra-assay and interassay coefficients of variation were <10% for each compounds.

Statistical Analysis

Values are expressed as mean ± SD, unless otherwise stated. Statistical analysis was performed by using 1-way analysis of variance and the Fisher exact test. Bonferroni’s adjustment to a P value was applied when appropriate. Correlations in 2 variables were determined with Pearson’s cor-
relation (r) coefficient analysis. Findings of $P < .05$ were considered to be significant.

**RESULTS**

All 63 girls were born at term (40.1 ± 0.65 weeks; 95% CI, 39.9–40.3) adequate for gestational age (Table I). At the study start, no significant difference was detected in CPP groups ($P > .05$; group A versus group B) and in control subjects ($P > .05$; group C versus group D).

Except for ZEA and α-ZOL, studied mycotoxins were undetectable in female subjects. At diagnosis, 6 girls (35%) with CPP (Figure; available at www.jpeds.com) had higher ZEA and α-ZOL levels than other groups, and 11 of 17 girls (65%) with CPP from Viareggio had undetectable mycotoxin levels ($P = .0217$, 2-tailed Fisher exact test). Their α-ZOL level was 106.5 ± 1.9 pg/mL (95% CI, 104.5–108.5), and the mean ZEA value was 933.7 ± 200.3 pg/mL (95% CI, 723.5–1143.9). By contrast, ZEA and its metabolites were not detected in 15 girls with CPP from Pisa (group B) at diagnosis and in control subjects (groups C and D) during routine evaluations. Furthermore, all 32 girls with CPP (groups A and B) had undetectable mycotoxin levels after 12 months of GnRH treatment.

Growth rates of 6 girls who were mycotoxin-positive with CPP (group E) were compared with 26 girls who were mycotoxin-negative with CPP (group F) and with 31 healthy control subjects (Table II). At diagnosis, no difference in chronological age (CA) and in target Ht-SDS was detected among the three groups. Also, BA and BA/CA ratio were not different in CPP groups before and after 12 months of TR treatment ($P > .05$, group E versus group F).

However, group E and group F exhibited different growth trends during TR treatment. At diagnosis, Ht-SDSCA and Wt-SDSCA were similar in CPP groups ($P > .05$, group E versus group F) and significantly higher than control subjects ($P < .05$ for both CPP series). Then, group E Ht-SDSCA significantly increased from baseline during TR treatment ($F = 5.121$; $P < .05$ at 12 months), and Ht-SDSCA slightly declined in group F ($P > .05$ for both time points versus baseline). Likewise, Wt-SDSCA increased from baseline in group E ($F = 5.145$; $P < .05$ at 12 months), but not in group F ($P > .05$ for both time points), respectively. Furthermore, higher Ht-SDSCA was observed in group E than in group F both at 3 months ($F = 2.626$; $P < .05$) and at 12 months of TR treatment ($F = 4.192$; $P < .01$), and Wt-SDSCA differed in groups with CPP only at 12-months ($F = 3.915$; $P < .01$, group E versus group F). Ht-SDSCA correlated with Wt-SDSCA in group E ($r = 0.986$, $P < .001$), and in group F ($r = 0.994$, $P < .001$). Both groups with CPP had higher Ht-SDSCA ($F = 8.217$, $P < .01$ for group E; $F = 1.960$, $P < .05$ for group F) and Wt-SDSCA ($F = 7.942$, $P < .01$ for group E; $F = 2.028$, $P < .05$ for group F) than control subjects after 12-months. Finally, BMI did not differ in groups with CPP at diagnosis and after 12 months ($P > .05$).

After 12 months of therapy, Ht-SDSBA was higher in group E ($F = 3.042$, $P < .05$ versus group F), although no difference was detected at the time of diagnosis ($P > .05$, versus group F). This is mainly because of an Ht-SDSBA increase in group E ($F = 5.165$, $P < .05$ versus group E baseline), and no change was detected in group F ($P > .05$ versus group F baseline).

During the entire follow-up period, HV-SDSCA was higher in group E (0.5 ± 0.75) than in both group F ($F = 2.777$, $P < .05$) and control subjects ($F = 4.977$, $P < .01$). HV-SDSCA in group E pronouncedly decreased after diagnosis; HV-SDSCA was 0.8 ± 0.68 and 0.4 ± 0.30 at 0- to 3-month and at 3-to 12-month intervals, respectively ($F = 5.137$, $P < .05$). By contrast, HV-SDSCA in group F was relatively constant during treatment and not different than its baseline.

At diagnosis, serum ZEA levels correlated with Ht-SDSCA ($r = 0.906$; $P < .05$), Wt-SDSCA ($r = 0.887$; $P < .05$), and Ht-SDSBA ($r = 0.821$; $P < .05$), and no correlation was detected for α-ZOL. At 12 months, no correlation was
detected between both mycotoxins and growth variables (P < .05 for Ht-SDS<sub>CA</sub>, Wt-SDS<sub>CA</sub>, HV-SDS<sub>CA</sub>, and Ht-SDS<sub>BA</sub>). Furthermore, BA and BA/CA did not correlate with ZEA or α-ZOL both at diagnosis and at end of follow up. No difference was found in groups with CPP at diagnosis and during TR treatment for luteinizing hormone, follicle stimulating hormone, and E<sub>2</sub> (P > .05, group E versus group F).

**DISCUSSION**

ZEA is a non-steroidal mycotoxin produced by Fusarium species on several grains. Despite its low acute toxicity and carcinogenicity, estrogenic ZEA also exhibits anabolic properties. ZEA food contamination is caused either by direct contamination of grains, fruits, and their products or by “carryover” of mycotoxins in animal tissues, milk, and eggs after intake of contaminated feedstuff. α-ZAL, a resorcyl lactone derived from ZEA, has been widely used as a growth promoter in the United States since 1969 to improve fattening rates in cattle. Its application was banned in the European Union since 1985. This also includes a ban on imported meat derived from cattle that given hormones for other than prophylactic and therapeutic purposes.

In vivo metabolism of mycoestrogens have been investigated in several animal species and human data are limited. ZEA undergoes reduction of 6-keto group to predominantly produce α-ZOL, the more active metabolite largely produced in humans, and β-ZOL. Then, ZEA and both ZOL-diasteroisomers are converted into α-ZAL.
and β-ZAL. Anabolic α-ZAL is predominantly metabolized into β-ZAL and, to a minor extent, into ZEA.24

The harmful effects of ZEA may be increased through its derivatives, α-ZOL and β-ZOL. ZEA metabolites mimic estrogens, acting as estrogen receptor agonists.13-15 With an in vitro assay, the most potent estrogen was α-ZOL, which had about the same potency than E2, and ZEA was at least 2 orders less potent than α-ZOL.13 Furthermore, in contrast to endogenous hormones, ZEA metabolites exhibit limited or no-binding to carrier proteins, allowing their easier access to estrogen target sites32; their potential potency is much larger than their actual concentrations suggest (as many as 50 times).14

The increased CPP frequency reported in the Viareggio countryside suggests possible estrogen pollution.16 In this view, we focused on dietary exposure to naturally occurring xenoestrogens such as ZEA and its congeners.

Of our 63 subjects, ZEA and α-ZOL were detected in 6 patients with CPP, all from the Viareggio countryside. Although previously suspected but never assayed,7,8,10,11 high mycoestrogen contamination was first determined in subjects with CPP. Detected concentrations of ZEA and α-ZOL in our subjects could represent high estrogenic tone, perhaps to a lesser extent than previously reported.12,30 These mycoestrogens could pose a risk to humans and especially to pre-pubertal children when endogenous E2 concentration is very low. Moreover, although this finding might be incidental, it may be related to CPP occurrence in girls exposed to mycoestrogens. Similar data were also reported for α-ZAL implants in heifers.33 However, ZEA pollution could not explain the epidemic CPP data of the Viareggio area, suggesting that other environmental factors such as dioxins and pesticides may be involved.

Comparing growth rates of groups with CPP, an anabolic effect of ZEA was suspected. During 12 months of TR treatment, patients who were mycotoxin-positive had a higher growth rate than patients who were mycotoxin-negative, which was in accordance with previous data.34 On the contrary, who were mycotoxin-positive were taller and proportionally heavier. In cattle, zeranol/α-ZAL implants improved growth rate and feed conversion efficiency. Anabolic treatments increased protein gain, reducing fat depositions in heifers.25-28 In girls who were mycotoxin-positive, a strong correlation between Ht-SDS CA and Wt-SDSCA, such as unchanged BMI, suggested an anabolic increase of linear growth improving lean mass gain over fat deposits.

Lampit et al35 showed that a prepubertal dose of estrogen replacement (8 μg/day conjugated equine estrogen) during TR-treatment (3.75 mg/4 weeks) in girls with CPP was effective for at least 24 months in maintaining reference range HV of about +1.0 SDS without acceleration of bone maturation higher than a ΔBA/ΔCA of 1 or pubertal development. The used mini-dose of estrogen was based on an attempt to replace prepubertal estrogen levels at least 5.0 pmol/L.35 The aforementioned data support that the administration of exogenous estrogens during GnRHa treatment improves growth rate in children with CPP.

Furthermore, patients who were mycotoxin-positive had a higher BA than control subjects, although not significantly different than patients who were mycotoxin-negative at diagnosis and after 12 months. In cattle, mycoestrogen-based treatments increased skeletal maturity compared with untreated control subjects.25 Although ZEA is stored in adipose depots and repeated exposure would result in a build up of mycotoxin, a single oral dose of tritiated ZEA showed a half-life of 22 hours in human blood.32 In this view, girl mycoestrogen exposure may be time-limited. Thus, incidental mycoestrogen exposure could induce central maturation of HPG axis and then, gonadal E2 levels mainly determined BA, such as in patients who are mycotoxin-negative, at diagnosis and during TR-induced hypogonadism. This hypothesis is supported by substantial BA maintenance and HV-SDSCA reduction during the follow-up period, such as by negative detection of mycoestrogens at 12 months of GnRHa treatment.

In conclusion, this study suggests a possible relationship between environmental mycoestrogen exposure and the development of CPP in females. In addition, these findings also point to anabolic growth effects of mycoestrogens in exposed girls.

The authors thank Federico Soldani, (McLean Division of Massachusetts General Hospital) for his scientific support.

REFERENCES

**Figure.** Living location of 6 girls who are mycotoxin-positive with CPP (full squares).