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Urinary zearalenone levels in girls with premature thelarche and idiopathic central precocious puberty

A. ASCI [1, 2], E. DURMAZ [3], P. ERKEKOGLU [1], D. PASLI [1], I. BIRCAN [3], B.KOCER-GUMUSEL [1]

[1]*Hacettepe University, Faculty of Pharmacy Department of Toxicology, Ankara, Turkey*
[2]*Atatürk University, Faculty of Pharmacy Department of Toxicology, Erzurum, Turkey*
[3]*Department of Pediatric Endocrinology Akdeniz University School of Medicine Antalya, Turkey*

Aim. **Recently, it was reported that the development of breast tissue and secondary sex characteristics in girls occurred at much younger age and the incidences of premature thelarce (PT) and central idiopathic precocious puberty (ICPP) are increasing. In this context, we wanted to evaluate the mycoestrogen exposure as triggering factor for premature sexual development.**

Methods. **The girls living in Mediterranean region of Turkey were divided in to three groups: control (N.=25; mean age: 6.45±1), PT (N.=28; mean age: 6.86±0.95) and ICPP (N.=25; mean age: 6.97±0.87). Urinary ZEN levels were measured by using ELISA technique and were normalized by urinary creatinine levels. Body Mass Index (BMI) was evaluated and sex hormone levels were also measured.**

Results. **We found that urinary ZEN was detectable in ~81% of all samples and observed an increase of ~2-fold in PT and a significant increase ~2.8-fold in ICPP group vs. control. We did not find any significant correlations between urinary ZEN levels and BMI and sex hormones in any of the groups.**

Conclusion. **To our knowledge, this is the first study evaluating urinary ZEN levels in PT and ICPP Turkish patients. We can postulate that ZEN exposure can contribute to the etiology of PT and PP; however further studies on large number of subjects are needed to confirm the present data.**

KEY WORDS: Zearalenone - Puberty, precocious - Sex characteristics.

Corresponding author: B.Kocer-Gumusel, Hacettepe University, Faculty of Pharmacy, Department of Toxicology, 06100 Ankara, Turkey.
E-mail: bgiray@hacettepe.edu.tr

Since the 1970s, there has been a worldwide interest on potential health hazards of human exposure to endocrine disrupting chemicals (EDCs), particularly estrogen disruptors. Many environmentally persistent compounds are either estrogen agonists, androgen antagonists, or both. Thus, they can disrupt the hypothalamic-pituitary-gonadal (HPG) axis, potentially inducing premature thelarche (PT) or idiopathic central precocious puberty (ICPP).[1]

The zearalenones (ZENs) are biosynthesized through a polyketide pathway as secondary estrogenic metabolites by many *Fusarium* species, including *Fusarium graminearum* (teleomorph *Gibberella zeae*). All these species are regular contaminants of cereal crops worldwide.[2] ZEN (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone), is a biologically potent, yet hardly toxic compound. Rather, it resembles 17b-estradiol (E_2), the principal hormone produced by the human ovary. Its structure allows it to bind to estrogen receptors (ERs) in mammalian target cells.[3]

ZEN can be better classified as a non-steroidal estrogen or mycoestrogen Animal studies show that dietary concentrations of ZEN as low as 1 ppm may lead to hyperestrogenic syndromes as well as infertility, abortion, disrupted conception or other breeding problems.[4-5] The reduced form of ZEN, zearalenol, has increased estrogenic activity. ZEN has also been used to treat postmenopausal symptoms in women, and both zearelanol and ZEN have been patented as oral contraceptives.[6, 7]

A number of studies were conducted showing a relationship between EDCs and early development in girls in recent years. In 1979, Fara *et al.* (1979) described a school epidemic of PT 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.[8] After this episode, the European Union banned the application/use of anabolic growth promoters in agriculture in 1985.[9, 10] In a study conducted on young Puerto Rican girls, 41 serum samples from PT patients and 35 control samples were analyzed. Significantly high levels of phthalates and their metabolites were identified in 28 (68%) samples from PT patients.[11] In Taiwan, it has been found that girls with PT had significantly higher levels of monomethyl phthalate (96.5±134.0 ng/mL) than the normal controls (26.4±30.0 ng/mL). The results suggest phthalates may be one of the contributing factors for early puberty in Taiwanese girls.[12] However, there are not many studies in literature concerning the effects of ZEN on premature sexual development. From 1978 to 1984, an epidemic of PT and PP occurred in Puerto Rico. It was suggested that dairy and meat products could be contaminated with anabolic estrogens such as zeranol or diethylstilbesterol, which were used for increasing muscle mass in cattle and poultry.[13-15] Schoental (1983) suggested the possibility of *Fusarium* toxin contamination of grain products as the causative agent.[16] It has been claimed that the high frequency of PT in Puerto Rico might be due to high serum ZEN levels; however no significant difference between case and control samples were found with a screening assay.[17] 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 PT patients with serum ZEN levels varying from 18.9 mg/L to 103 mg/L.[18] Moreover, a study conducted on the sera obtained from PP patients suggested a possible relationship between environmental ZEN exposure and the development of PP in North-West Tuscany (Italy).[19] In literature, there is only one study in which the ZEN levels of healthy New Jersey girls were measured and the researchers found that girls with mycoestrogen-positive urine were less likely to have reached the onset of breast development.[20]

Taking into account all the available knowledge and data, the aim of this study was to determine urinary levels of ZEN in PT and ICPP patients living in Mediterranean region of Turkey and to evaluate the mycoestrogen exposure as triggering factor for premature sexual development.

Materials and methods

Chemicals and kits

All the chemicals used in the study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glucuronidase/aryl sulfatase was from Roche (Mannheim, Germany). RIDASCREEN® Zearalenon kits and RIDA C18 columns was purchased from R-Biopharm (Darmstadt, Germany). All HPLC equipments were obtained from Agilent (Santa Clara, CA). Commercial kits for luteinizing hormone (LH), follicle-stimulating hormone (FSH) and E_2 were purchased Roche (Mannheim, Germany).

Subjects

All the subjects (ages 4-8 y) were living in Antalya province, a city in Mediterranean Region of Turkey. Subjects were admitted to Akdeniz University Pediatric Endocrinology

Department in Antalya between September 2010 and February 2012. The study composed of 3 groups: control group (Control; N.=25; mean age: 6.45±1); girls with PT (PT, N.=28; mean age: 6.86±0.95) and girls with ICPP (PP, N.=25; mean age: 6.97±0.87).

All the subjects employed in the study were non-obese. Obesity was defined as Body Mass Index (BMI) above 95th percentile according to our national standards.[21]

Control group consisted of girls with no history of PT or PP and without any known endocrinologic disorder; pathology and endocrine disease and these girls had no secondary sexual characteristics in their physical examination. Besides, girls in the control group were not using any pills or creams. In the physical examination, ICPP was defined as the presence of both breast development and pubic hair with onset of thelarche after the forth year of life but before the eighth year. Children with a diagnosis of peripheral PP, a history of exogenous exposure to estrogen containing pills or creams, or benign non-progressive PT and family history of ICPP were excluded from the study. PT was defined as the presence of only breast development with no other sexual characteristics. At their examination, basal LH, FSH and estrogen levels, bone age and pelvic sonography findings were evaluated. All patients were scanned with central MR for excluding intracranial pathologies. Luprolide stimulation test was applied to all the patients. LH≥5 IU/L is considered to be diagnostic for ICPP. When LH<5 IU/L and thelarce is evident for almost 1 y, the patients were employed to PT group.

Morning urine samples were collected from both control and patient groups and and blood samples were obtained only from the PT and ICPP patients. The samples were aliquoted and stored in -20 °C and then transferred to Hacettepe University Faculty of Pharmacy, Department of Toxicology on dry ice. Samples were kept in -20 °C until analysis.

All subjects participated in the study voluntarily and written consent was obtained from the parents of the children involved and the study was approved by Akdeniz University Ethical Committee according to the Declaration of Helsinki.

Determination of urinary zearalenone levels

Urine ZEN levels were measured by using a "Zearalenon ELISA kit". The extraction of ZEN was done according to the manufacturer's instructions. Briefly, urine samples were spiked with 50 ppt ZEN before starting the experiments. 0.5 mL urine sample was diluted with 3 mL of sodium acetate (50 mM). 8 mL of glucuronidase/aryl sulfatase (from *Helix pomatia*) was added on the mixture, in order to obtain free ZEN from ZEN glucuronide and ZEN sulfate metabolites. The mixture was incubated at 37°C for 3 h. Before loading the samples on C18 column, column was rinsed with 3 mL methanol (100%). The column was equilibrated with 2 mL Tris buffer (pH:8.5)/ methanol (80:20, v/v) mixture. Urine sample (3.5 mL) was loaded to column (flow rate 1 drop/second) and the column was rinsed with 3 mL methanol (40%). Column was dried for 1 min under nitrogen stream. Later, samples were eluted with 1 mL methanol (80%) (flow rate 15 drops/min). The eluent was dried under nitrogen stream. All the dried eluents were kept in -20 °C until analysis.

For ZEN determination, dried eluent was dissolved in 50 mL methanol and mixed with 450 mL of sample dilution buffer. Standards were at 50, 150, 450, 1350 and 4050 ppt concentrations. The ELISA protocol was applied according to manufacturer's instructions. Briefly, 50 ml of sample or standard and 50 ml of enzyme conjugate (peroxidase conjugated ZEN) was added to each well. The plate was incubated at room temperature for 2 h on a shaker. Wells were emptied and washed. Later, 50 mL of substrate (urea peroxide) and chromogen (tetramethylbenzidine) were added to each well and plate was incubated at room temperature for 30 min on a shaker. Stop solution (1 N sulfuric acid) was added to each well and the optical density (OD) of the samples was read at 450 nm. Special software, the RIDA©SOFT

Win, was used for evaluation of ZEN levels in the samples.

The detection limit of the kit was 50 ppt for urine samples. The recovery rate of the method was 106.80±9.43%. The urinary creatinine values were determined by high pressure liquid chromatography (HPLC).[22] Results were given as pg ZEN/g creatinine.

Determination of serum hormone levels

Serum hormone levels (LH, FSH and E_2) were measured by electrochemiluminescence immunoassay (ECLIA) using commercial kits.

Statistical analysis

The differences among the groups were evaluated with Kruskal-Wallis test, followed by Mann Whitney U test. The correlations between ZEN and BMI, LH, FSH and E_2 were assessed by Spearman's Rho (ρ) correlation coefficient. All the statistics were performed by using a Statistical Package for Social Sciences Program (SPSS) version 17.0. P-values <0.05 were considered as statistically significant. The results are expressed in mean±SEM.

Results

BMI

The BMI was calculated by dividing the weight to the square of the height. Concerning BMI, we did not find any statistical difference between C and PT (P=0.60), as well as between C and ICPP (P=0.88).

Urinary zearalenone levels

We found that ZEN was detectable in ~81% of all the urine samples. In control group, the mean urinary ZEN level was found to be 103.63±14.38 pg/g creatinine while in PT group it was 191.98±43.62 pg/g creatinine and in PP group, it was detected as 288.92±56.18 pg/g creatinine (Table I). We have observed that the urinary ZEN levels of PT girls were ~2-fold higher and urinary ZEN levels of ICPP girls were ~2.8-fold higher (P<0.05) compared to control. Because of the high standard deviation in PT group, the change was not found be statistically significant (P=0.07).

Correlations between body mass index, sex hormones and urinary zearalenone levels

The correlations between ZEN levels and BMI and sex hormones are given in Table II. We did not find any significant correlations between urinary ZEN levels and BMI in any of the groups (ρ between control and PT groups is -0.077 and ρ between control and ICPP groups is 0.169). Although we found a 31.3% negative correlation between basal FSH and urinary ZEN levels in PT group, the correlation was not found to be statistically significant. On the other hand, we observed a 38.9% positive correlation between basal FSH and urinary ZEN levels in ICPP patients; however the correlation was not significant (P>0.05). Although there was a positive correlation between urinary ZEN levels and basal E_2 levels in PT group (ρ=0.151, P>0.05), we observed negative correlation between urinary ZEN

TABLE I.—*The urinary ZEN levels in the study groups.*

	Number of samples		ZEN (pg/g creatinine)			
	Detectable	Non-detectable	Min	Max	Median	Mean±SEM
Control	22	3	78.58	233.98	115.06	103.63±14.38 [a]
PT	25	3	56.56	804.11	99.59	191.98±43.62 [a, b]
ICPP	16	9	140.84	810.92	263.22	288.92±56.18 [b]

ZEN levels are given in pg/g creatinine.
[a, b] Columns that do not share same letters (superscripts) are significantly different from each other (P<0.05).
ZEN: Zearelenone; PT: premature thelarche; ICPP: idiopathic central precocious puberty

TABLE II.—*Correlations between BMI, LH, FSH, E_2 and ZEN levels in the study groups.*

	Spearman's Rho (ρ)	
	PT	PP
BMI-ZEN	-0.077	0.169
Basal LH-ZEN	0.092	-0.045
Basal FSH-ZEN	-0.313	0.389
Basal E_2 -ZEN	0.151	-0.434

Spearman's Rho (ρ) is used a correlation coefficient.
P<0.05 values are considered as statistically significant.
No significant correlations were observed between ZEN levels and BMI, basal LH, basal FSH and basal E_2 levels.
ZEN: Zearelenone; BMI: Body Mass Index; LH: luteinizing hormone, FSH: follicle stimulating hormone; E_2: 17-b estradiol

levels and basal E_2 levels in ICPP group (ρ=-0.434, P>0.05)

Discussion

Puberty is as a critical and special time in a girl's development during which environmental exposures, particularly to EDCs, can have a major effect on risk of developing breast cancer in the future.[1] PP is defined by the development of secondary sexual characters before the age of 8 years. Precocious breast development is usually due to the premature activation of the hypothalamo-pituitary-ovarian axis, defining ICPP. It may also correspond to PT, which is defined by non-pathological isolated early breast development.[23] In the recent years, a steep increase in ICPP and PT cases has drawn major concern over EDCs. Estrogenic or anti-androgenic EDCs like phthalate esters, bisphenol A (BPA), and dichlorodiphenyltrichloroethane (DDT) have been the subject of various animal research as well as several epidemiological studies.[24] These compounds are thought to capable of affecting onset and progression of puberty

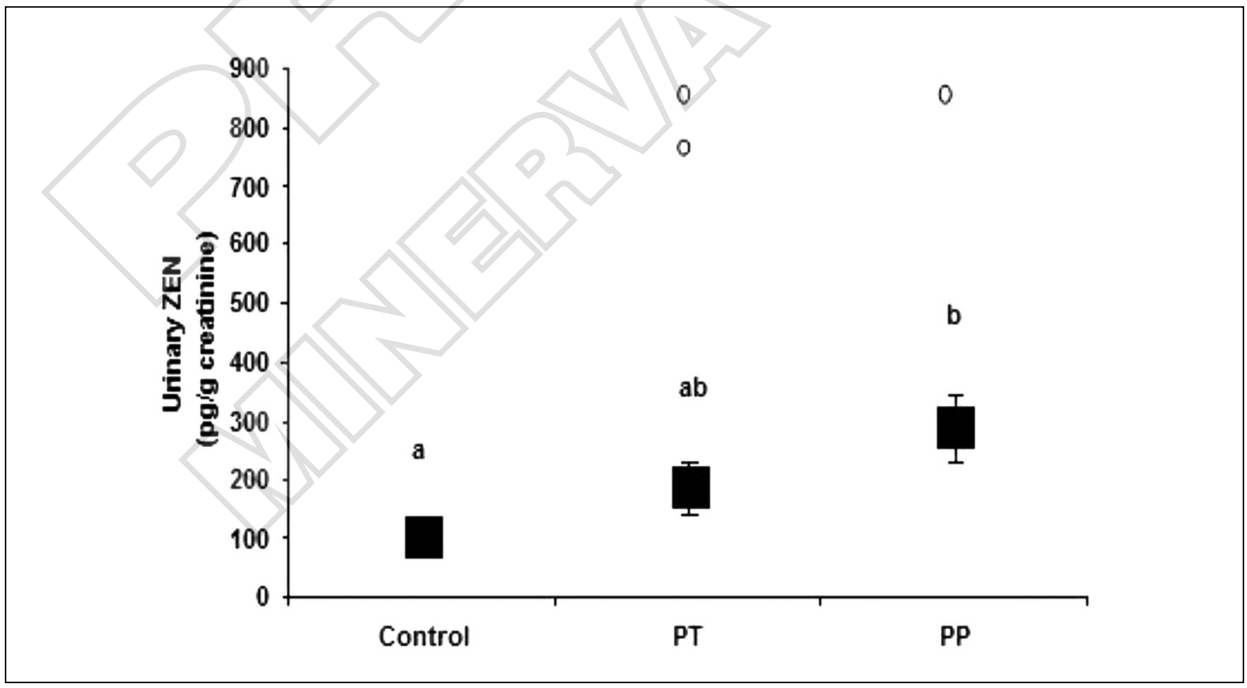

Figure 1.—ZEN levels in the study groups. ZEN levels are given in pg/g creatinine.
The results are expressed in mean±SEM.
ZEN: Zearelenone; Control Group (N.=25); PT: premature thelarche group (N.=25); ICPP: idiopathic central precocious puberty group (N.=28)
[a, b] Bars that do not share same letters (superscripts) are significantly different from each other (P<0.05).

as well as breast development in both boys and girls.[25] The mechanism underlying the advancement of puberty caused by EDCs has been postulated to involve an acceleration of hypothalamic maturation.[26] Our recent studies on PT and PP patients revealed that urinary BPA levels and urinary mono(2-ethylhexyl)phthalate (MEHP) levels were higher in both PT and PP patients when compared to control girls.[27]

ZENs are mycotoxins, present in both grains and other plant foods through fungal contamination by *Fusarium* species, and in animal products (*e.g.*, dairy products, meat, eggs). Their acute toxicity is low so these compounds cannot be classified as toxins, but rather can be described as phytoestrogens, mycoestrogens, and growth promoters because of their estrogenic activity.[7, 28] ZEN has been shown to bind to both to estrogen receptor α (ERα) and estrogen receptor ß (ERß), acting as a full agonist for ERα and a mixed agonist-antagonist for ERß, exhibiting much higher binding affinity than that found for other well-known EDCs, such as BPA, or DDT, in both receptor subtypes.[29] On the other hand, it is not clear how much the ZEN contributes to the total environmental load of xenoestrogens.[30]

The purpose of this study was to evaluate urinary levels of ZEN, as well as its relationship with BMI, LH, FSH, and E_2 in PT and ICPP patients. To our knowledge, this is the first study evaluating ZEN levels in Turkish girls. We chose to measure total ZEN levels in the urine. We found that ZEN was detectable in a large proportion of participants (81%) which is in line with earlier reports.[19, 20] Surprisingly, we found little knowledge in literature on the health effects of ZENs in girls, particularly their impact during puberty, a period highly sensitive to estrogenic stimulation. Although there are several anecdotal reports of epidemics of ICPP, which were attributed to the use of anabolic estrogens in animal foods, such as zeranol, in Italy [8] and Puerto Rico,[13, 14, 16, 17] the levels of ZENs were not actually measured. As mentioned above, serum mycoestrogens levels were assessed in small clinical studies of PP in Hungary,[18] Puerto Rico,[17] Italy [19] and in United States.[20] In the Italian study, mycotoxin-positive girls were taller and proportionally heavier than those who were mycotoxin-negative. However, the researchers did not observe any difference in the basal LH, FSH and E_2 levels between ZEN positive PP girls and ZEN negative PP girls.[19] However, in the American study, girls having mycoestrogen-positive urine were significantly of shorter stature and less likely to have reached the onset of breast development after adjusting for age, BMI, isoflavone intake, and recruitment year. Fat mass, waist circumference, hip circumference and BMI were all insignificantly lower in girls who were mycoestrogen-positive. Besides, high mycoestrogen positive girls tend to have less BMI than the negative and low mycoestrogen positive girls.[20] In the present study, we did not find a difference between the BMIs of control girls and girls with PT or ICPP. Thus, our study populations consisted of non-obese girls. Besides, no correlations were observed between the measured hormone levels and ZEN levels in any of the groups. We have to indicate that this study was performed on relatively low number of subjects. Therefore, our findings need to be replicated in larger studies, so that the role of mycoestrogens on the onset of early thelarche, early puberty and pubertal markers can be fully understood. Besides, researchers must consider that the estrogenic or antiestrogenic effects of ZEN seem to depend on dose, hormonal environment, age, and critical window of administration.[19, 20]

If we compare the uncorrected ZEN levels (without dividing urinary ZEN levels to urinary creatinine) in Turkish girls to American girls, we can observe that mean ZEN levels of Turkish control group is 75±62.14 pg/mL (median 65.51 pg/mL), whereas for American girls, the levels were 1820±480 pg/mL (median 380 pg/mL).[20] This shows that American girls are 5.8-fold more exposed to ZEN when compared to Turkish girls and we can indicate that in healthy subjects, ZEN levels are dramatically high in United States compared to Turkey. In our previous studies, we have observed that

population living in Mediterranean region of Turkey highly consumed vegetables and fruit. This might be the underlying factor for the big difference in urinary ZEN levels between American girls and Turkish girls.[31, 32]

ZEN levels in foodstuffs are not yet regulated in many parts of the world.[2] Extensive reviews of Canadian and Scandinavian epidemiological data have concluded that the risk of ZEN to human populations is minimal. The recommended safe human intake of ZEN is estimated to be 0.05 mg/kg body weight per day.[3] Questions have been raised about the rationale used by governments to regulate ZEN and about the implementation of guidelines in different countries.[33, 34] The only country that has provided a rationale for setting limits for mycotoxins (other than aflatoxins) in human foods and animal feeds is Canada, where risk assessments have been performed for deoxynivalenol, ZEN, and ochratoxin A.[34, 35] Turkey has set limits to ZEN levels in cereals, and to additional baby food in Turkish Food Codex by a statement (Statement 2008/26). In cereals (except maize), the maximum limit (ML) is set as 100 mg/kg, whereas in maize, the ML is 350 mg/kg, in maize farina, it is 200 mg/kg and the ML in corn oil is 400 mg/kg. The ML is additional baby food is quite low and is set as 20 mg/kg.[36] However, limits have to be set in meat, dairy products and in eggs as well as in feedstuff. In many parts of the world including United States, the ML limits were not set in food or feed, perhaps because the acute toxicity of ZENs is thought to be quite low.

As another part of the current study, we also measured other EDCs in the same patients and the results show that other EDCs (BPA, DEHP and its metabolites) are also found higher in the urine of PT and ICPP patients (unpublished data). Concerning the whole frame, we can postulate the combined effects of EDCs should be the main concern of researchers in the future rather than the effect of one single EDC. Despite these effects, data in humans are surprisingly lacking, given the fact that mycoestrogens are present in our food supply as much or more than isoflavones are, which have been studied extensively. Literature shows that the combined effects can be observed as additivity between the same classes of EDCs like between two/more estrogenic or anti-androgenic compounds.[37, 38] On the other hand, the combination effects between different classes of EDCs make this subject more complex and inextricable. Therefore, enhancing basic and clinical research on these substances and on their mixtures is needed in order to understand their mode of action deeply.[39] Given the fact that ZEN is not regulated in feed or foodstuff in different parts of the world, the potential health effect of this particular mycoestrogen needs to be elucidated. For the regulation of EDCs, involvement of individual and scientific society stakeholders is necessary for communicating and implementing changes in public policy and awareness.

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