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OPINION OF THE

SCIENTIFIC COMMITTEE ON FOOD

ON

FUSARIUM TOXINS

PART 2¹: ZEARELENONE (ZEA)

22 JUNE 2000

¹ The present opinion deals only with ZEA and therefore does not address all the terms of reference outlined below for fusarium toxins in general. The Committee will address the general aspects when all the individual toxins have been considered.

Terms of reference

Although it is acknowledged that there are gaps in the toxicological information available, the Scientific Committee on Food is requested

- to assess the health risk associated with exposure to the different Fusarium toxins in cereals, taking into the account the current state of knowledge.
- to indicate, on the basis of current knowledge, which of these Fusarium toxins are of most concern for public health and for which there is an urgent need for further research and/or need for measures to reduce the presence of these toxins in cereals.
- to indicate, if possible, the nature of the toxicological studies to recommend in order to elucidate (more) completely the toxicology of these toxins.

In considering these issues the Committee is asked to take note, inter alia, of the comprehensive report "Fusarium toxins in cereals – a risk assessment" which has been prepared for the Nordic Council of Ministers.

Background

A variety of Fusarium fungi, which are common soil fungi, produce a number of different mycotoxins of the class of trichothecenes (T-2 toxin, HT-2 toxin, deoxynivalenol (DON)) and nivalenol and some other toxins (zearalenone and fumonisins). The Fusarium fungi are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found on cereals grown in the temperate regions of America, Europe and Asia. Fusarium toxins have been shown to cause a variety of toxic effects in both experimental animals and livestock. On some occasions toxins produced by Fusarium species have also been suspected to cause toxicity in humans.

Introduction

In the evaluation of Fusarium toxins the criteria for toxin selection have been:

- the toxins most commonly found in analytical surveys of cereals
- the toxins for which there is a minimum of toxicological data.

The first group of toxins to be evaluated is: deoxynivalenol (SCF 1999), T-2 toxin, HT-2 toxin, nivalenol, fumonisin B1 and zearalenone.

The present evaluation deals with Zearalenone (ZEA); it is primarily based on the monograph prepared for the 53th meeting of the Joint FAO/WHO Expert Committee on Food Additives in June 1999 (JECFA 2000), a report prepared for the Nordic Council of Ministers "Fusarium toxins in cereals – a risk assessment" (Eriksen and Alexander, 1998), a detailed review of 339 publications on physicochemical data, isolation and purification, analytical methods, mycology, laboratory and natural production, occurrence and stability in foods and feeds as well as on its toxicity by Kuiper-Goodman *et al.* (1987) and a monograph of the International Agency for Research on Cancer (IARC 1993).

JECFA (2000) concluded that the safety of zearalenone could be evaluated on the basis of the dose that had no hormonal effects in pigs, the most sensitive species. JECFA established a provisional maximum tolerable daily intake (PMTDI) for zearalenone of 0.5 µg/kg of body weight. This decision was based on the NOEL of 40 µg/kg of body weight per day obtained in a 15-day study in pigs (see below). The Committee also took into account the lowest observed

effect level of 200 µg/kg body weight per day in this pig study and the previously established ADI of 0-0.5 µg/kg body weight for the metabolite α -zearalanol, evaluated as a veterinary drug (JECFA 1998). The Committee recommended that the total intake of zearalenone and its metabolites (including α -zearalanol) should not exceed this value.

Zearalenone (ZEA)

Description

ZEA is a nonsteroidal estrogenic mycotoxin produced by several *Fusarium* species. It has been implicated in numerous mycotoxicoses in farm animals, especially in pigs. It is found worldwide in a number of cereal crops such as maize, barley, oats, wheat, rice and sorghum (Kuiper-Goodman *et al.* 1987, Tanaka *et al.* 1988), and also in bread (Aziz *et al.* 1997). ZEA was shown to be produced on corn by *Fusarium* isolates from the continents of Australia, Europe, and North America (Vesonder *et al.* 1991) as well as in New Zealand (diMenna *et al.* 1997) and South East Asia (Philippines, Thailand, and Indonesia; Yamashita *et al.* 1995). The occurrence of ZEA in food and feed was also demonstrated in South America (Dalcero *et al.* 1997, Molto *et al.* 1997), Africa (Doko *et al.* 1996), Taiwan, China and the U.S.S.R. (Ueno *et al.* 1986). *Fusarium* isolates from bananas were also shown to be able to produce ZEA (Jiménez *et al.* 1997).

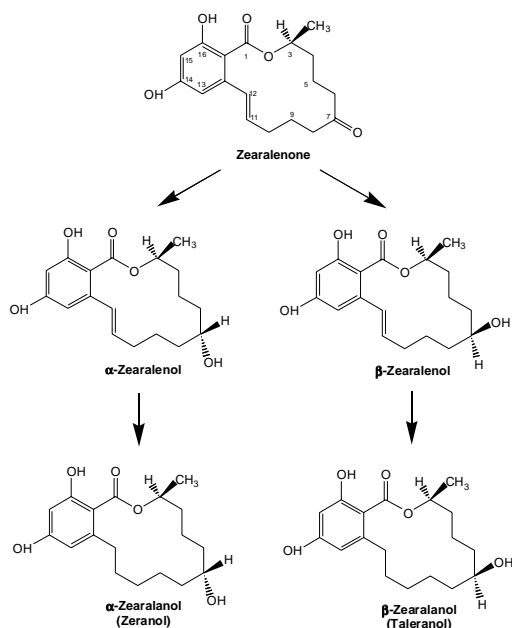
Chemistry

Zearalenone: 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1,7(8*H*)-dione, C₁₈H₂₂O₅, MW: 318.36, CAS 17924-92-4.

ZEA is a stable compound, both during storage/milling and the processing/cooking of food, and it does not degrade at high temperatures. Wet milling of corn concentrates ZEA in the gluten fraction (2-7 fold concentration) (Kuiper-Goodman *et al.* 1987, Gilbert, 1989, Lauren and Ringrose, 1997).

Toxicokinetics

Studies of pharmacokinetics and metabolism indicate that ZEA is fairly rapidly absorbed following oral administration and can be metabolised by intestinal tissue in pigs and possibly in humans during its absorption, with the formation of α - and β -zearalenol and α - and β -zearalanol, which are subsequently conjugated with glucuronic acid. Quantitatively, glucuronide conjugates of the parent compound and α -zearalenol predominates in these species. Differences between species in the metabolism of ZEA were found: In the rat, most of the zearalenone is found as free zearalenone or glucuronide conjugate thereof, while only small amounts of zearalenols and their conjugates are formed (Kuiper-Goodman *et al.* 1987, JECFA 2000).



Biliary excretion with entero-hepatic circulation occurs in rats and mice, while urinary excretion predominates in rabbits. Urinary excretion is also the main route of excretion in pigs in spite of the demonstrated entero-hepatic circulation of ZEA, owing to a high degree of reabsorption in the gut. The very limited human data (one individual) suggest that urinary excretion is also significant in humans (Kuiper-Goodman et al. 1987).

In humans, as in pigs, ZEA was mainly found in urine as glucuronide conjugates of the parent compound and α -zearalenol (Kuiper-Goodman et al. 1987, JECFA 2000).

Biochemical mode of action

ZEA and some of its metabolites have been shown to competitively bind to estrogen receptors (ER) in a number of in vitro systems. Binding to specific receptors has been demonstrated in uterus, mammary gland, liver and hypothalamus from different species. Values for ER binding in target tissues and cells relative to estradiol (E2) of ZEA were between < 0.01 – 0.1 whereas α -zearalanol showed somewhat stronger binding and β -zearalanol much less binding. The relative binding affinities to the rat uterine cytoplasmic receptor for ZEA and derivatives were α -zearalanol $>$ α -zearalenol $>$ β -zearalanol $>$ ZEA $>$ β -zearalenol (Kuiper-Goodman et al. 1987, Eriksen and Alexander, 1998).

ZEA was shown to bind and activate both the ER α and ER β in cells transfected with human ER α and ER β . For ER α , ZEA was found to be a full antagonist and for ER β to be a mixed agonist-antagonist (Kuiper et al. 1998).

The potency of ZEA relative to E2 in the mouse uterotrophic assay upon oral administration was about 0.001. The potency relative to E2 in the mouse vaginal cornification assay of ZEA was 0.001 and 0.01 upon subcutaneous and topical administration, respectively. α -zearalanol had

about the same level of potency in this assay, but is usually severalfold more active in the uterotrophic assay (Kuiper-Goodman et al, 1987).

In blood, ZEA and zearalanol bind to human sex hormone-binding globulin to some extent (Kuiper-Goodman et al. 1987, Eriksen and Alexander, 1998).

Toxicity

Acute toxicity

ZEA has low acute toxicity after either oral or interperitoneal administration in mice, rats and guinea pig (oral LD50 values of >4000 up to >20,000 mg/kg b.w., Kuiper-Goodman et al. 1987, JECFA 2000).

Subacute and Subchronic toxicity

In oral toxicity studies of up to 90 days, the effects seen in experimental as well as in domestic animals appeared to be dependent on interactions of ZEA or its metabolites with the ER. Pigs and sheep appear to be more sensitive than rodents; in controlled studies with well-defined exposure to multiple doses, the NOEL in pigs was 40 µg/kg of body weight per day on the basis of estrogenic effects in responsive tissues and reproductive performance (see Reproductive and developmental toxicity for details), compared with a NOEL of 100 µg/kg body weight in rats (see Chronic toxicity and carcinogenicity for details) (Kuiper-Goodman et al. 1987, JECFA 2000).

Chronic toxicity and carcinogenicity

B6C3F₁ mice were fed diets containing 0, 50 or 100 mg/kg ZEA for 103 weeks (males: 0, 8 or 17, females: 0, 9 or 18 mg/kg b.w./day). No significant difference in survival or changes in body weight gain was observed between groups. Treatment-related non-neoplastic lesions were not found in male mice. In females, estrogen-related, dose dependent effects were seen in several tissues (fibrosis in the uterus, cystic ducts in mammary glands), as well as myelofibrosis in the bone marrow. Hepatocellular adenomas were found in 8, 6 and 14% and 0, 4 and 14% in males and females, respectively. The increase was statistically significant in the high-dose females. A statistically significant trend was observed in the incidence of pituitary adenomas in both males (0, 9, and 14%) and females (7, 5, and 31%) Pituitary carcinomas were found in one male in the low-dose group and in two females in the high-dose group. However, the incidence of pituitary carcinomas in treated and control animals was not statistically significantly different. (NTP, 1982).

FDRL Wistar rats were fed dietary ZEA for 104 weeks (0, 0.1, 1.0 or 3.0 mg/kg b.w./day). Significantly increased liver weights were found in male and females exposed to 3.0 mg/kg bw, and uterine weights were increased in females in the two highest treatment groups. In rats receiving the highest dose of ZEA, increased trabeculation of the femur was noted. Apart from that, no biologically significant changes were seen, and no treatment-related tumours were found (Becci *et al.*, 1982). Survival rates and tumour incidences were not reported. A NOEL of 0.1mg/kg b.w./day can be taken from this study.

Fischer 344 rats were fed diets containing 0, 25 or 50 mg/kg ZEA for 103 weeks (0, 1, or 2 mg/kg b.w./day). Mean body weight gains of treated rats were lower than those of controls, and the depression in mean body weight (by 19 % in males and 11 % in females in the high-dose group after 44 weeks of exposure) was dose-related. No significant difference in survival was observed between groups. The following non-neoplastic lesions were observed: inflammation of the prostate gland, testicular atrophy, cysts or cystic ducts in mammary glands of males, increased incidence of hepatocellular cytoplasmic vacuolization in males, and an increased incidence of chronic progressive nephropathy in both sexes at both doses. Retinopathy and cataracts were observed in increased incidence in low- and high-dose males, and in low-dose females. No treatment-related increase in tumour incidence was found in the study (NTP, 1982).

ZEA was evaluated by the International Agency for Research on Cancer (IARC) in 1993. Based on inadequate evidence in humans and limited evidence in experimental animals ZEA was allocated, together with other Fusarium toxins, in Group 3 (not classifiable as to their carcinogenicity to humans).

Genotoxicity

ZEA has been tested for genotoxicity in a number of test systems (Kuiper-Goodman *et al.* 1987, IARC 1993, Ghedira-Chekir *et al.*, 1998). ZEA did not induce SOS error-prone DNA repair in *E. coli*, while it induced differential toxicity in *B. subtilis* repair-deficient and -proficient strains (*rec* assay): ZEA did not induce gene mutations in *S. typhimurium* (Ames test) or mitotic crossing over in *S. cerevisiae*. It induced sister chromatid exchanges (SCE), chromosomal aberrations and polyploidy in Chinese hamster ovary cells *in vitro* in the absence of exogenous metabolic activation. SCE was weakly induced in cultured human lymphocytes.

DNA-adduct formation

In female BalB/c mice, treated i.p. with a single dose of ZEA (2 mg/kg b.w.), several different DNA-adducts in the liver and the kidney were detected by ³²P-postlabelling. Co-administration of α -tocopherol (4 mg/kg bw) significantly decreased DNA-adduct formation (Grosse *et al.* 1997).

Weanling female Sprague-Dawley rats were fed a diet containing 0.05 mg/kg ZEA (5 μ g/kg b.w.) for three weeks. ZEA did not induce any DNA adduct formation (measured by ³²P-postlabelling) in liver-, kidney- and uterus-DNA (Li *et al.* 1992)

12-15 different DNA adducts were found in the kidney and liver of female BalbC mice treated with a single dose of ZEA (2 mg/kg b.w. i.p. or orally) using a ³²P-postlabeling method. The total DNA adduct levels were higher in liver compared to kidney and also higher after i.p. treatment compared to oral treatment. In mice ovary, 6 different DNA adducts appeared only after repeated doses (1 mg/kg b.w. on days 1, 5, 7, 9 and 10). In contrast, no DNA adducts could be detected in male and female Sprague-Dawley rat organs after i.p. treatment (no details given, Pfohl-Leszkowicz *et al.* 1995).

Immunotoxicity

Several alterations of immunological parameters were found at high ZEA concentrations *in vitro* (inhibition of mitogen-stimulated lymphocyte proliferation, increased IL-2 and IL-5 production). (Eriksen and Alexander, 1998, JECFA, 2000).

B6C3F₁ mice were fed a diet supplemented with 10 mg/kg ZEA (1.5 mg/kg b.w./day) for 2 weeks. After i.v. infection with *Listeria monocytogenes* the splenic bacterial count showed an increasing trend on days 1 and 4 compared with the control animals (11 animals). No negative effects were seen after 8 weeks of feeding. The exposure did not affect the splenic plaque-forming response to sheep red blood cells or the delayed hypersensitivity response to keyhole limpet haemocyanine after either 2 nor 8 weeks (Pestka *et al.*, 1987).

Zearalenol was subcutaneously administered to female B6C3F₁ mice at a dose of 45 mg/kg b.w.. The animals were subsequently infected with *Listeria monocytogenes*. No differences in survival or bacterial enumeration in spleen could be seen between animals exposed to ZEA as compared to control animals (Pung *et al.*, 1984).

In female B6C3F₁ mice fed a diet supplemented with 10.0 mg/kg ZEA (1.5 mg/kg b.w./day) for 6 weeks, no differences from the control group could be seen in serum concentration of IgG, IgM or IgA or in white blood cell count or in differential counts (lymphocytes, polymorphonuclear neutrophils, monocytes and eosinophils) (Forsell *et al.*, 1986).

Reproductive and developmental toxicity

ZEA causes alterations in the reproductive tract of laboratory animals (mice, rat, guinea-pigs, hamsters, rabbits) and domestic animals. Various estrogenic effects like decreased fertility, increased embryo-lethal resorptions, reduced litter size, changed weight of adrenal, thyroid and pituitary glands and change in serum levels of progesterone and estradiol have been observed but no teratogenic effects were found in mice, rats, guinea pigs and rabbits (Kuiper-Goodman *et al.* 1987, JECFA, 2000). Pigs and sheep appear to be more sensitive than rodents.

There are several published experimental studies with pigs, the pivotal one being a study with sexually mature non-pregnant gilts which were given 2 kg of feed containing 0, 1, 5 or 10 mg/kg ZEA in the diet between day 5 and day 20 of oestrus (0, 40, 200 or 400 µg/kg b.w./day). The inter-oestrus interval increased significantly from 21.0 ± 0.3 days in the control group to 29.2 ± 2.9 and 32.7 ± 3.3 days in gilts fed 5 or 10 mg/kg ZEA in the diet. The inter-oestrus interval was not affected in gilts given 1 mg/kg in the diet. Increased plasma concentrations of progesterone and prolonged maintenance of corpora lutea was observed in gilts with prolonged cycles. The corpora lutea regressed when ZEA was withdrawn from the diet (Edwards *et al.*, 1987). A NOEL of 40 µg/kg b.w./day can be taken from this study.

In a limited study using a low number of prepubertal female pigs², estrogenic effects were reported at lower doses (Bauer *et al.*, 1987). 2 pigs were fed 0.25 mg/kg ZEA in the diet (equivalent to 10 µg/kg b.w./day) for 11 days, followed by 5 days of feed without ZEA. 2 additional pigs were fed 0.05 mg/kg ZEA (equivalent to 2 µg/kg b.w./day) for 21 days, and only one pig was used as a control animal. The higher dose resulted in redness and swelling of

² According to a personal communication with Bauer (March 2000), prepubertal pigs and pure ZEA were used in this study, in contrast to other studies on pigs in which contaminated feed was given containing potentially not only ZEA but also other estrogenic contaminants.

the vulva, swelling of the mammarys, and numerous vesicular follicles and some cystic follicles were seen on the ovaries. With the low dose, no external changes were seen at the end of the experimental period, but autopsy showed that the number of vesicular follicles on the ovaries was higher in treated animals than in the control animal. These reported effects would require confirmation using larger numbers of animals to establish a clear NOEL.

Effects in humans

ZEA was measured in endometrial tissue from 49 women. There were 27 endometrial adenocarcinoma, 11 endometrial hyperplasia and 11 normal proliferative endometrium with ZEA values of 47.8 ± 6.48 , 167 ± 17.69 ng/ml, and below detection limit in the groups respectively. In 8 cases of hyperplastic and 5 cases of neoplastic endometrial tissue ZEA was not detected (Tomaszewski et al, 1998).

ZEA or zearalanol was suspected to be the causative agent in an epidemic of precocious pubertal changes in young children in Puerto Rico between 1978 and 1981 (Sàenz de Rodriguez, 1984, Sàenz de Rodriguez et al., 1985). ZEA or metabolites were detected in blood plasma. The authors reported high responses in cytosol receptor assay with rat uterus when testing homogenates of locally produced meat, indicating the presence of substances binding to the ER. Later studies by FDA failed to detect any of the estrogen growth promoter used in food (anon. 1986). Natural sources of estrogen-acting compounds, like some plant metabolites or mycotoxins as the cause have not been ruled out. Later, a statistical correlation between the pubertal changes and consumption of both meat products and soy-based formula was found. However, the statistical associations could not explain more than 50% of the investigated cases and the authors suggested that better diagnosis and reporting or some unsuspected factor could count for the reported increase in premature thelarche in girls between 6 months and 8 years (Freni-Titulaer et al., 1986).

Increased incidence of early telarche has been reported from the south-east region of Hungary. ZEA was found in concentrations from 18.9-103.5 $\mu\text{g/ml}$ in serum sampled from the patients and ZEA was also present in samples of surplus food collected from the patients (Szuets et al., 1997). No details are given.

Evaluation and Conclusion

Hepatocellular adenomas and pituitary tumours were observed in long-term studies of carcinogenicity in mice. However, these tumours were observed only at doses greatly in excess of the concentrations that have hormonal effects i.e. at levels 8-9 mg/kg of body weight or more. The Committee concluded that these tumours are a consequence of the estrogenic effects of ZEA. A similar conclusion was drawn by JECFA (1988) in the evaluation of α -zearalanol. In rats, there was no treatment-related increase in the incidence of tumours at doses of 1 – 3 mg/kg of body weight per day. ZEA did not induce gene mutations in bacteria or recombination in yeast. However, ZEA induced SCE and chromosomal anomalies *in vitro*, and DNA-adducts as measured by ^{32}P -postlabelling in mice but not in rats. The presently available data on genotoxicity of ZEA do not allow an adequate evaluation of its genotoxic potential and its mechanism of action in inducing chromosomal anomalies and DNA-adducts in mice.

The Committee concluded that the safety of ZEA could be evaluated on the basis of the dose that had no hormonal effects in pigs, the most sensitive species, and established a temporary

TDI for ZEA of 0.2 µg/kg of body weight. This decision was based on the NOEL of 40 µg/kg of body weight per day obtained in a 15-day study in pigs and the lowest observed effect level of 200 µg/kg body weight per day in this study. A safety factor of 200 was used because it is a temporary TDI due to some deficiencies in the data base (e.g. the question of a higher sensitivity of prepubertal vs adult pigs raised by new information from the study of Bauer et al. 1987).

Estimates of average dietary intakes of ZEA based on the five FAO regional diets range from 1.5 to 3.5 µg/day (for “European” and “Middle Eastern” diets, respectively). Assuming a mean body mass of 60 kg, these intakes correspond to 0.03 and 0.06 µg/kg of body weight per day respectively. Estimates of average dietary intakes of ZEA based on individual diet records are <0.98 µg/day (0.02 µg/kg bw per day) for Canada, 1.2 µg/day (0.02 µg/kg bw per day) for Denmark, 1.1 µg/day (0.02 µg/kg bw per day) for Norway and <2.1 µg/day (0.03 µg/kg bw per day) for the US.

Needs / suggestions for future studies

Additional studies to determine the no hormonal effect level in prepubertal pigs should be performed (See Bauer et al 1987).

Additional studies are needed on the potential genotoxicity of ZEA, especially to clarify its ability to bind covalently to DNA.

There is a need for comparative studies on species differences (including use of human cells) in the metabolism of ZEA to elucidate the differential findings in long term carcinogenicity studies in rats and mice with respect to tumour formation as well as the difference between rats and mice with respect to DNA-adduct formation as measured by the ³²P-postlabeling method.

Studies on blood levels of ZEA in humans with known dietary ZEA-intake could help to clarify the toxicokinetic behaviour of ZEA.

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