IN VIVO ASSESSMENT OF ZEARALENONE TOXICITY

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ABSTRACT

The microscopic filamentous fungi of the genus *Fusarium* are capable of producing secondary metabolites—mycotoxins. *Fusarium* fungi synthesize trichothecenes, zearalenone (ZEA) and fumonisins under appropriate environmental conditions. In this biological experiment, we studied the effects of zearalenone on a model organism called *Artemia franciscana*. During the three-day *in vivo* tests, we used five different concentrations of zearalenone (0.08 ppm, 0.4 ppm, 2 ppm, 10 ppm and 50 ppm). The results of this study showed that as the zearalenone concentration and the duration of the mycotoxin exposure increased, the lethality of *Artemia* also increased. Our study showed that the toxicity of zearalenone to *Artemia franciscana* was relatively low.

Key words: *Artemia franciscana*; *in vivo*; toxicity; zearalenone

INTRODUCTION

Zearalenone (ZEA) is one of the most important mycotoxins and it is produced by the *Fusarium* toxigenic species: *F. graminearum*, *F. culmorum*, *F. equiseti*, *F. crookwellense*, *F. roseum*, *F. nivale*, *F. tricinctum*, *F. sporotrichioides*, *F. oxysporum*, *F. moniliforme*, *F. lateritium*, *F. sacchari*, *F. sambucinum*, *F. gibbosum* and others [2, 14]. Zearalenone (Fig. 1) is a non-steroidal estrogenic mycotoxin that has a structure similar to steroid hormones, increasing its ability to bind to the intracellular oestrogen receptors of the uterus, hypothalamus and pituitary gland. It acts as an agonist and partly as an estradiol antagonist. As a result of this action, zearalenone inhibits the secretion of the follicle-stimulating hormone (FSH) and suppresses the maturation of ovarian follicles in the preovulatory phase [20]. In terms of toxicity, the ZEA is responsible for the functional changes in the reproductive system with subsequent overall disruption of hormonal regulation. In addition to its estrogenic effects, it may also be hepatotoxic, haematotoxic, immunotoxic and genotoxic [34].

Fig. 1. Chemical structure of zearalenone

Chemically, zearalenone is a macrocyclic lactone of β -resorcylic acid and has a close structural relationship with other antibiotic metabolites that are produced by a number of microscopic filamentous fungi [6]. Zearalenone is a white crystalline substance with a melting point of 159–163 °C. Its chemical name is 6-(10-hydroxy-6-oxo-trans-1-undecenyl) β -resorcylic acid lactone. The molecular formula of zearalenone is $C_{18}H_{22}O_5$ and the molecular weight is $318.36 \text{ g}\cdot\text{mol}^{-1}$. ZEA is a relatively stable substance that is not subject to degradation in the cereal processing or heat treatments. It is soluble in benzene, acetonitrile, methanol, ethanol and acetone [8].

Effects of ZEA on the domestic animals

Pigs are particularly sensitive to ZEA compared to other animal species, because their liver undergoes a biotransformation of zearalenone and consequently the synthesis of α -zearalenol, which is more toxic than zearalenone alone. Compared to zearalenone and β -zearalenol, α -zearalenol has a higher ability to bind to oestrogen receptors [19]. As early as in 1927, a disease called Zearalenone-syndrome was observed in pigs after the feeding of mouldy corn. The main symptoms of the Zearalenone-syndrome in pigs included changes in the genital system such as oedema and vulvar inflammation, vaginal and rectal prolapse and enlargement of the mammary gland [9]. Gimeno and Quintanilla [10] reported a case of zearalenone intoxication flare up on a horse farm. Among the most observed symptoms in mares were feed rejection, uterine prolapse and internal haemorrhages. In males, in turn, a sharp weakening of the genital apparatus was observed. Various sources have reported that the cases associated with zearalenone intoxication in ruminants were mainly related to its

estrogenic effect and included, in particular, abortions in females [16, 17, 22]. Other symptoms include: vaginitis, increased vaginal secretion, decreased reproductive capacity, and enlarged mammary glands in heifers [32]. In poultry, in addition to neck oedema, ovarian cysts have also been observed [1]. In young turkeys, comb oedema, inflammation of the anus and enlargement of the ovaries have been described. The influence of libido and adverse effects on spermatogenesis has been observed in ganders and cocks, and fertility has been reduced in laying hens [9]. In dogs and cats, a local contact mucosal irritation has occurred following zearalenone intoxication. Upon transfer of zearalenone to the body, mycotoxin acts as a false estrogen, causing acute vulvovaginitis, disturbances of the estrous cycle and impaired fertility [30]. The estrogenic effects of ZEA caused several reproductive disorders in animals, as well as hyperestrogenic syndromes in humans [24].

Occurrence of zearalenone in feed and food

Zearalenone is worldwide [2] and contaminates about 32 % of cereals and their products (biscuits, breakfast cereals, pastries, desserts) [4]. It is found mainly in corn, oat, rye, rice, sorghum and wheat [5, 33].

Zearalenone classification according to IARC

According to the International Agency for Research on Cancer (IARC), zearalenone belongs to group No. 3, which includes mycotoxins that are not classified as carcinogenic to humans [15]. However, zearalenone is currently covered by the Commission Regulation (EC) No. 1881/2006, which sets the maximum levels for certain food contaminants.

MATERIALS AND METHODS

We used commercial zearalenone (Sigma Aldrich, Schnelldorf, Germany) for experimental purposes. The biological activity of zearalenone was monitored *in vivo* on a model invertebrate organism—*Artemia franciscana*. The conditions for the optimal hatching of artemias were prepared according to Dvořák et al. [7]. The natural environment of *Artemia franciscana* is the inland salt water; so it was necessary to prepare “seawater”, the chemical composition of which is given in Table 1.

Dried cysts “Maxima brine shrimp eggs” (Sanders, Utah, USA) were added to the prepared seawater. Favour-

Table 1. Chemical composition of the seawater

Chemicals	Weight [g.l ⁻¹]
NaCl	23.900
MgCl ₂ .6H ₂ O	10.830
CaCl ₂ .6H ₂ O	2.250
KCl	0.680
Na ₂ SO ₄ .10H ₂ O	9.060
NaHCO ₃	0.200
SrCl ₂ .6H ₂ O	0.040
KBr	0.099
H ₃ BO ₃	0.027

able hatching conditions were ensured by the sufficient oxygen supply and constant egg movement, using a Maxima vibrating membrane compressor (Hagen, USA). The optimal hatching rate of artemia was achieved according to Sanders instructions in 18 hours at a temperature of 27–30 °C, a salinity of 1.2–3.0 ‰ and a pH of 7.5–8.5. Since hatching was performed at 25 °C, it was extended to 24 hours.

Zearalenone toxicity test procedure

In the three-day toxicity tests, we used the methodology of Dvořák et al. [21]. For each test, we used 10 nauplii stages of *Artemia franciscana*, which were transferred to a Petri dish (60 mm diameter) containing 10 ml of seawater using a Pasteur pipette. The toxic effects of 5 concentrations of zearalenone (50 ppm; 10 ppm; 2 ppm; 0.4 ppm; 0.08 ppm) were observed. Since zearalenone is not water soluble, DMSO was used for dilution; the final concentration in seawater being 1 %. Each zearalenone concentration was tested on a set of 50 individuals, divided into five Petri dishes (à 10 nauplii). The viability of artemia was compared to the two control groups. The first control group (50 artemias divided into 5 Petri dishes of 10 individuals in 10 ml of seawater) served as a workflow control. A second control group (50 individuals divided into 10 pieces of 5 petri dishes) was placed in 10 ml of sea water solution containing 1 % DMSO (DMSO effect control). A constant temperature of 22 °C was ensured by placing the samples in

a thermostat during the experiments. The survival of artemia was observed after 24, 48 and 72 hours. We considered living artemia to be individuals that demonstrated movement compared with those that did not move any part of their body. The viability of artemias was observed using a magnifying glass against a black background.

Statistical evaluation

The data obtained in this study were reported as means ± standard deviation (SD) and analysed using ANOVA and the Dunnett's test. The statistical significance among parameters was considered at $P < 0.05$.

RESULTS

By using the *in vivo* toxicity test of zearalenone, we observed its toxic effect on the model organism of the *Artemia franciscana*. The lethality of artemia induced by different concentrations of zearalenone, as a function of exposure time is presented in Table 2. The results of the statistical analysis showed that as the zearalenone concentration and the duration of exposure increased, the lethality of artemia also increased.

Compared to the control group (0 ppm), a significant increase in the lethality of artemia was observed after a 24-hours exposure to zearalenone at 10 ppm (16 %, $P < 0.001$) and 50 ppm (12 %, $P < 0.001$). After a 48 h exposure, a statistically significant death of artemia was observed at concentrations of: 0.4 ppm (8 %, $P < 0.01$), 2 ppm (10 %, $P < 0.001$), 10 ppm (16 %, $P < 0.001$) and 50 ppm (18 %, $P < 0.001$). Also, after 72 hours, the lethality of artemias was significantly increased at zearalenone concentrations of: 0.4 ppm (8 %, $P < 0.05$), 2 ppm (12 %, $P < 0.01$), 10 ppm (18 %, $P < 0.001$) and 50 ppm (24 %, $P < 0.001$).

The determination of LD50 (Probit Analysis) at the tested zearalenone concentrations was not possible due to the low lethality of *Artemia*.

DISCUSSION

At present, an *in vivo* test on the aquatic invertebrates of the genus *Artemia* has been used to verify the toxic effects of various substances. This assay was designed by Michalel et al. in 1956 [21]. The model organism for re-

Table 2. The lethality [%] of *Artemia franciscana*

ZEA [ppm]	24 h		48 h		72 h	
	x ± SD	L [%]	x ± SD	L (%)	x ± SD	L [%]
0	10.0 ± 0	0	10.0 ± 0	0	10.0 ± 0	0
0.08	10.0 ± 0	0	10.0 ± 0	0	9.6 ± 0.55	4.0
0.4	9.4 ± 0.55	6.0	9.2 ± 0.45 **	8.0	9.2 ± 0.45 *	8.0
2	9.4 ± 0.55	6.0	9 ± 0 ***	10.0	8.8 ± 0.45 **	12.0
10	8.4 ± 0.55 ***	16.0	8.4 ± 0.55 ***	16.0	8.2 ± 0.45 ***	18.0
50	8.8 ± 0.84 **	12.0	8.2 ± 0.45 ***	18.0	76 ± 0.55 ***	24.0

ZEA—zearalenone; x—mean number of live individuals; SD—standard deviation; L—lethality of artemia; significance of differences compared to control sample: *— $P < 0.05$; **— $P < 0.01$; ***— $P < 0.001$

search fields such as environmental ecotoxicology, genetics, and evolutionary studies continues to be *Artemia* spp. [3, 23]. The principle of these tests is based upon the lethality of *Artemia* after the treatment with the test substance as a function of concentration and exposure time [25]. It is used to test the biological activity of plant extracts [31], cyanobacterial toxins [13] and has previously served as a screening test for heavy metal toxicity [29], pesticides [21] and fungal mycotoxins [12].

To assess the elemental toxicity of hazardous substances under *in vivo* conditions, a bioassay using artemia aquatic animals is currently preferred [11, 18], because it is a simple and inexpensive test using a large number of organisms for statistical validation and no special equipment is needed [25]. Biological assay with artemia does not require animal serum, therefore it prevents unnecessary use of animals in scientific experiments [26].

The best studied of the *Artemia* species is *Artemia franciscana*. It represents the vast majority of studies in which *Artemia* is used as an experimental test organism [28]. Our results of toxicity testing of zearalenone on the model organism *Artemia franciscana* indicated a relatively low level of zearalenone toxicity. The highest lethality (24%) was seen after 72 hours exposure to zearalenone at a concentration of 50 ppm. Similarly, the low lethality of artemia (18%) was also observed by H a r w i n g and S c o t t [12], who used a zearalenone concentration of 10 ppm in their experiments. However, when tested for the toxic effect of fumonisin B2 at 66 ppm, H a r t l and H u m p f [11] found nearly 100% lethality of artemia after 48 hours

exposure. According to R i c h a r d s o n and H a m i l t o n [27], the brine shrimp assay is suitable for the prediction of trichothecene toxicity in broiler chicks. The low lethality of *Artemia franciscana* after exposure may be explained by the fact that zearalenone, as a mycotoxin with estrogenic effects, primarily binds to oestrogen receptors and is responsible for functional changes in the reproductive system, with subsequent disruption of hormonal regulation [20, 34].

CONCLUSIONS

The occurrence of secondary metabolites of microscopic filamentous fungi is a global problem. They cause humans and animal mycotoxicosis. Zearalenone causes functional changes in the reproductive system, therefore constant investigations of this toxic substance is essential. This study pointed to the low lethality of the model organism *Artemia franciscana*. Further biological experiments are needed to determine the effects of zearalenone on the reproductive apparatus of *Artemia* spp.

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