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Original article

Influence of zearalenone on selected biochemical parameters in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Zearalenone (ZEA) is a mycoestrogen frequently found in food and animal feed materials all over the world. Despite its hydrophobic character, ZEA is also found in surface and ground waters which suggests an environmental risk for aquatic animals. Knowledge concerning mycotoxin-related mechanisms of toxicity is still incomplete, e.g. little is known about the influence of ZEA exposure on fish. The aim of this study was to investigate the effect of ZEA on selected biochemical parameters in juvenile rainbow trout after 24, 72, and 168 h of intraperitoneal exposure (10 mg/kg of body weight). The analysis showed a slight tendency towards prolonged blood clotting time and significant iron deficiency in the liver and ovary of exposed animals. However, no differences in aminotransferase (AlaAT, AspAT) activity or glucose levels in fish plasma was observed. The results of this study suggest that although trout exposed to ZEA did not exhibit any distinct symptoms of liver damage, the mycotoxin tested was able interfere with blood coagulation and iron-storage processes.

Key words: blood coagulation, fishes, iron deficiency, liver enzymes, plasma glucose

Introduction

Zearalenone (ZEA) is a nonsteroidal mycotoxin, produced by various fungi of the *Fusarium* genus, which are frequently found in cereal crops and other plant products all over the world, resulting in contamination of food and animal feed material (Gajęcki 2002, Fink-Gremmels 2008). The structure of ZEA (6-[10-hydroxy-6-oxo-E-1-undecenyl]-B-resorcyclic acid lactone) is chemically stable, making the mycotoxin resistant to storage, milling, food processing, and cooking (Lauren and Smith 2001). Despite the hydrophobic character of the molecule, ZEA was also found in surface and ground waters (Gromadzka et al.

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2009, Gajęcka et al. 2011) which suggests a possible environmental risk for aquatic animals.

Numerous studies of the toxicity of ZEA reveal its various effects on animals and humans. However, the most studied aspect of this mycotoxin is its property to exert dysfunction or structural disorders around the reproductive tract of domestic animals (Zinedine et al. 2007). Due to this estrogenic potential, ZEA belongs to a group of chemicals classified as endocrine disrupting compounds (EDCs). The most crucial feature of the chemical structure of ZEA is its ability to bind to estrogen receptor (ER) and induce expression of estrogen responsive genes. Exposure to ZEA leads to a number of reproductive disorders in mammals, e.g. decreased libido, anovulation, infertility, or neoplasmic lesions, which all derive from the mechanisms that alter transcription of ER-dependent genes (Tiemann et al. 2003, Zinedine et al. 2007, Minervini and Dell'Aquila 2008, Jakimiuk et al. 2009). The few *in vivo* studies on fish revealed effects of ZEA and its metabolites on ER-dependent gene expression induction, immune system, growth, and reproduction (Arukwe et al. 1999, Kelev et al. 2002, Woźny et al. 2008, 2010, Schwartz et al. 2010).

While most of the biological properties of ZEA are attributed to the agonistic effect on the ER, the compound was found to produce certain biological responses that cannot be simply explained by its estrogenic activity. For example, in orally exposed rats, ZEA induced modifications of haematological and biochemical parameters, indicating liver toxicity and impairment of blood coagulation process (Maaroufi et al. 1996). In another experiment, lymphoid infiltration and focal inflammation were found in the liver and kidney of exposed mice (Abbès et al. 2006). The mechanisms that stand behind these effects are still to be fully understood, but it is unlikely that the overall toxicity of ZEA is solely due to its estrogenicity. In fact, ZEA was recently classified as a highly oxidant mycotoxin (El Golli-Bennour and Bacha 2011), suggesting that the oxidative damage may be the predominant toxic effect for ZEA action.

Despite advancements in understanding the mechanisms of mycotoxin toxicity, little is known about the toxic effects of ZEA exposure on aquatic vertebrates, fish in particular. The aim of this study was to investigate the influence of ZEA on selected biochemical parameters in juvenile rainbow trout after 24, 72, and 168 h of intraperitoneal exposure at a dose of 10 mg/kg. The effect was estimated based on selected physiological markers, such as aminotransferase activity (AlaAT, AspAT), plasma glucose level, blood clotting time, or iron concentration in the liver and ovary.

Materials and Methods

The fish were maintained in accordance with the regulations set forth by the Local Ethical Commission No. 64/2008 issued on 18th of September 2008 (conforming to principles of Laboratory Animal Care; NIH 1985). Juvenile all-female rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) individuals with an average body weight of 55.3 ± 5.3 g and length of 17.9 ± 0.5 cm were obtained from the Department of Salmonid Research in Rutki (Inland Fisheries Institute in Olsztyn, Poland). All fish were acclimated 2 weeks prior to exposure. During the housing period, the rainbow trout were held in 800 L flowthrough tanks (wellwater, 600 L/h; photoperiod of 14/10) at 7°C and fed four times a day to satiation. The trout were deprived of food 2 days prior to injections.

Randomly sampled individuals were anesthetized by immersion in etomidate solution (Kazuń and Siwicki 2001), and injected intraperitoneally with ZEA (at a dose of 10 mg/kg of body weight) dissolved in corn oil as a carrier solution, or corn oil alone (control sample). After 24, 72, or 168 h of exposure, five individuals ($n = 5$) were randomly taken from each of the experimental group, then were anesthetized and blood samples were collected for assessment of blood clotting time, aminotransferase activity, and plasma glucose level. After fish sacrifice, liver and ovaries were frozen (-20°C) for further iron content determination.

Both aminotransferase (AlaAT, AspAT) activities, and plasma glucose concentration were determined in blood taken from the caudal vein of each anestesized individual, as described in Brzuzan et al. 2009. Briefly, blood was stored in heparinized tubes (Polfa, Poland) and centrifuged at 4000 g for 20 min at 15°C. The supernatant (plasma) was frozen and stored at 80°C until further measurement. Plasmatic activity of AlaAT and AspAT was determined at $\lambda = 340$ nm (37°C) with Alpha Diagnostics kits (No. A6624, and A6661 respectively), according to the manufacturer's instructions. Results were expressed in unit per liter [U/L], where 1 U is the amount of enzyme that converts 1 mmol substrate per min under the specific conditions of the procedure. Plasma glucose concentration [mg/dL] was determined ($\lambda = 500$ nm; 37°C) with Alpha Diagnostics (Warsaw, Poland) kit No. G6620, according to the manufacturer's instructions.

To investigate any possible influence of ZEA on the blood coagulation process of the exposed fish, we used the method of Okrasa (2009) with slight modifications. Briefly, 20 μL of blood from the caudal vein was placed on a microscope slide, and a capillary glass tube ($\phi = 0.4$ mm; 150 mm length) was filled in a hori-

zontal position. A 5 mm long piece of blood-filled capillary was then removed (chipped off) at 15 s regular intervals until a blood clot was formed. The number of capillary fragments removed was multiplied by 15 s to obtain the time necessary for blood clot formation [s].

For iron content determination in the liver and ovaries of fish, frozen tissues were lab oven dried at 60°C for 48 h. The samples were then homogenized in a porcelain mortar and digested in a 3:1 mixture of concentrated hydrochloric (9 mL) and nitric acid (3 mL), following heating in a microwave oven (MarsXpress; CEM Corporation; USA) for 25 min at 170°C. Mineralized samples were filtered via Whatmann 40. filters to 50 mL volumetric flasks. The concentration of iron [mg/kg of dry weight] was measured using flame absorption spectrophotometry (AA 280 FS; Varian; Australia) at $\lambda = 248.3$ nm.

Differences in AlaAT and AspAT activities, plasma glucose, clotting time, and iron concentration among all experimental groups were estimated using one-way ANOVA followed by Tukey's HSD test. Additionally, *t*-test was used for pair-wise comparison (control vs. exposed) at the respective time-point. Levenne and Shapiro-Wilk tests were performed to fulfill the requirements for parametric test application. Data were calculated using Statistica 9 software (StatSoft; Tulsa, USA).

Results

Table 1 presents the results of liver enzyme activity, plasma glucose level, blood clotting time, and iron concentration in the liver and ovary of rainbow trout exposed to ZEA (10 mg/kg), compared to control at each time-point of the experiment (0, 24, 72, 168 h post application). AlaAT, AspAT activity, and plasma glucose levels were not significantly different between experimental groups and exposure time-points.

Interestingly, a slight tendency of prolonged clotting time was noted in the exposed group of fish. Blood clotting time of trout exposed to ZEA for 168 h was longer than fish exposed for a shorter period (i.e. 24 h) or those of the control group from earlier time-points (control: 0, 24 h; Tukey's HSD, $p < 0.05$). However, no differences in blood clotting time were observed in treated fish when compared to control at the respective time-point (*t*-test, $p > 0.05$).

After each exposure period (24, 72, 168 h), iron concentration in the liver and ovary of fish treated with ZEA was significantly lower in comparison to the respective control group (*t*-test, *p* values in Table 1), and the lowest concentration values were noted in the ovary at the end of the study (168 h; Table 1). Surprisingly, an apparent increase of iron concentration in the ovary of the control fish was observed after 24 h of the experiment (Tukey's HSD, $p < 0.05$).

Discussion

Several mycotoxins, such as aflatoxins or T-2 toxin, have been previously studied for induction of adverse effects in aquatic animals (Santacroce et al. 2008). Depending on the severity of the intoxication, fish reveal impaired swimming, anemia, liver damage, tumors, and blood coagulation alterations (Poston et al. 1982, Anh Tuan et al. 2002, Santacroce et al. 2008). However, knowledge concerning the biological properties of ZEA in fish is still insufficient. This is one of the very few (if not the first) studies documenting effects of short-term ZEA exposure on selected biochemical parameters in rainbow trout species.

Table 1. Enzyme activities (AlaAT and AspAT), plasma glucose, blood clotting time, liver and ovary iron concentration in rainbow trout injected with corn oil (Control) or 10 mg/kg of zearalenone (ZEA) for each time-point (0, 24, 72, 168 h) post application.

Parameter		Control				ZEA (10 mg/kg)		
		0 h	24 h	72 h	168 h	24 h	72 h	168 h
AlaAT [U/L]		$8.31^{a} \pm 1.43$	$11.99^{a} \pm 1.56$	$8.25^{a} \pm 2.39$	$10.70^{a} \pm 0.30$	$10.11^{a} \pm 3.50$	$9.85^{a} \pm 0.77$	$10.09^{a} \pm 0.21$
AspAT [U/L]		$281.33^{a} \pm 62.85$	$301.33^{a} \pm 103.73$	$369.83^{a} 75.33$	$502.00^{a} \pm 68.00$	$426.83^{a} \pm 126.88$	$536.67^{a} \pm 91.51$	$413.00^{a} \pm 90.93$
Plasma glucose [mg/dL]		$77.87^{a} \pm 4.40$	$109.00^{a} \pm 8.08$	$62.93^{a} 7.62$	$76.37^{a} \pm 0.07$	$106.17^{a} \pm 19.83$	$67.87^{a} \pm 7.89$	$71.87^{a} \pm 11.23$
Blood clotting time [s]		$138^{a} \pm 7$	$143^{a} \pm 10$	$171^{a,b} \pm 26$	$183^{a,b} \pm 10$	$141^{a} \pm 13$	$190^{a,b} \pm 14$	$240^{b} \pm 31$
Iron concentration [mg /kg]	liver	$191.9^{a} \pm 14.8$	$281.5^{b,c} \pm 9.2$	$292.1^{b,c} \pm 9.7$	$243.0^{a,c} \pm 14.4$	$234.0^{a,c*} \pm 15.0$	$190.8^{a***} \pm 8.1$	$196.0^{a**} \pm 6.8$
	ovary	$203.8^{a} \pm 11.2$	$291.3^{b} \pm 9.6$	$197.1^{a} \pm 9.9$	$198.1^{a} \pm 8.6$	$160.1^{a,c**} \pm 8.7$	$141.9^{a,c***} \pm 8.5$	$128.4^{c**} \pm 1.5$

Values are given as mean ±S.E. ($n = 5$). Different letters denote means that are significantly different for the respective values using one-way ANOVA followed by Tukey's HSD test ($p < 0.05$). Asterisks indicate significant difference of means for the control group at the respective time-point using *t*-test (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$).

Alterations of plasma glucose levels may occur due to various environmental or physiological events, e.g. handling stress, anesthesia, or bleeding (Heath 1995) but it works as a non-specific hallmark of disturbed physiology. On the other hand, activities of serum aminotransferases are widely considered to be sensitive indicators of hepatic injury (Pfeifer et al. 1977), as the hepatocyte damage alters membrane permeability and leads to the release of enzymes from the cells into the circulation system (Zimmerman and Seeff 1970). In the present study, 168 h of exposure to ZEA at a dose of 10 mg/kg did not cause any significant differences in AlaAT, AspAT activity or glucose levels in fish plasma among any experimental groups or examined time-points (Table 1).

Our results may be surprising, as there is a strong evidence that oxidative metabolism of ZEA impairs liver function and disturbs blood parameters, indicating the liver as the target organ of ZEA action in rodents (Maaroufi et al. 1996, Abbès et al. 2006, Stadnik and Borzęcki 2009) and other animals (Čonková et al. 2001, Šperanda et al. 2006, Jiang et al. 2011). However, it has become evident that the toxic effect of ZEA depends on the dose, time of exposure, animal species, and gender. For example, 14 days of oral exposure to ZEA at a dose of 100 μg/kg body weight resulted in a significant increase in AlaAT and AspAT activity in female rabbits (Čonková et al. 2001) but not in male rats (Stadnik and Borzęcki 2009). Since the literature concerning research on the biological properties of ZEA, available for fish, is limited, we postulate that the applied dose of ZEA (10 mg/kg) for 168 h of exposure was too low to exhibit liver injury in rainbow trout and influence the liver markers. This leads to the conclusion that the rainbow trout may be a much less sensitive species of the study of sub-acute toxicity of ZEA than laboratory rodents.

Several studies over the past decade report on the adverse effects of ZEA on animal hemostasis, mostly rodents. For example, intraperitoneal treatment with ZEA (5 mg/kg) impaired rat blood coagulation, expressed as a 3-fold increase in bleeding time and a 2-fold decrease in platelet count (Maaroufi et al. 1996). Liver inflammation and reduction of platelets was also observed in mice after oral exposure to ZEA at doses of 40 and 500 mg/kg (Abbès et al. 2006). To date, no similar study has been performed on fish species. It is known that blood coagulation may be influenced by many factors such as stress, liver damage, or exposure to different chemical compounds; and research on teleosts indicate that the process is fundamentally similar to that in mammals, but simpler (Tavares-Dias and Oliveira 2009). In the present study, we showed a slight tendency for prolonged blood clotting time in rainbow trout treated with ZEA (Table 1). We speculate that ZEA affected the rainbow trout blood coagulation process, similarly to its effect in mammals. However, we cannot rule out the possibility that the slight changes were caused by a secondary stress effect, i.e. fish handling or anesthesia (Barton and Iwama 1991). Application of standardized and more reliable methods for measurement of fish hemostasis would be useful in further investigations.

Interestingly, our results indicated a significant iron decrease in the liver and ovary of fish exposed to ZEA, when compared to the respective control group (Table 1). So far, no research has been performed on the influence of ZEA on iron deficiency in fish vital tissues. However, a recent study on EDCs, known to inflict oxidative stress in exposed organs (such as the liver and ovary), may help to understand the results obtained in our study. In the paper of Rashid et al. (2009), treatment of normally-nourished (iron-rich diet) rats with bisphenol A (bisA, a well-known xenoestrogen) resulted in iron deficiency with a concomitant decrease in antioxidant enzyme activity and increased effect of lipid peroxidation. Moreover, the deleterious influence of bisA was aggravated in animals maintained on an iron-deficient diet (anaemic rats). In addition, the effect was more pronounced in female gonads, suggesting ovaries being more susceptible to reactive oxygen species (Rashid et al. 2009). It seems that not only iron deficiency is a confounding factor in oxidative stress but oxidative stress may also be a reason for modulation of iron concentration. However, in the present state of knowledge concerning regulation of cellular iron content for fish species (which is scarce at best), the question of the exact mechanism responsible for observed iron deficiency remains as yet unanswered.

Surprisingly, a significant modulation of iron concentration was also observed after 24 h of the experiment in the ovary of control fish injected with corn oil alone (Table 1). Since corn oil is frequently used in toxicological studies as a vehicle solvent of lipophilic compounds (and is intended to be neutral for experimental animals), we believe that the apparent increase in iron concentration could be also produced by handling stress or fish anesthesia (Barton and Iwama 1991).

In conclusion, the results of this study suggest that although trout exposed to ZEA did not exhibit any distinct symptoms of liver damage, the mycotoxin tested was able interfere with blood coagulation and iron-storage processes.

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