EXPRESSON OF CYPscc AND 3B-HSD mRNA IN BITCH OVARY AFTER LONG-TERM EXPOSURE TO ZEARALENONE

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Abstract

In the presented study, sexually immature beagle bitches were exposed per os to 50 or 75 µg/kg b.w. of zearalenone (ZEA) for 42 d, and real-time qPCR assay was performed to investigate a possible effect of the treatment on CYPscc and 3β-HSD mRNA expression in canine ovary. While the exposure of bitches to ZEA at a lower dose (50 µg/kg b.w.; 100% NOAEL) did not cause distinct effect on the expression of examined genes, the treatment with the higher dose (75 µg/kg b.w.; 150% NOAEL) resulted in over 5-fold increase in 3β-HSD mRNA level (P<0.05). The results suggest that the appearance of ZEA in bitches ovaries may trigger the accumulation of 3β-HSD in a way similar to that of pregnenolone. Further, more detailed experiments need to be performed in order to assess whether ZEA and its major metabolites, α- and β-zearalenol, are involved in transcriptional regulation of the key steroidogenic enzymes.

Key words: bitches, ovary, zearalenone, mRNA, qPCR, steroidogenesis.

Zearalenone 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 feed (4, 7, 20). In the past few years, a potential health risk for accompanying pets became of growing problem in pet feed industry that may use plant products contaminated with significant concentrations of mycotoxins (1, 21). Carnivore accompanying animals (e.g. cats or dogs) seem to be more vulnerable to adverse effects of mycotoxins than the other pets, as their organisms are not evolutionary prepared for the occurrence of undesirable substances (like ZEA) in their feed. Therefore, chronic exposures by feeding, even with relatively low doses of mycotoxins, may result in serious health implications for the animals, or even death. For example, long-term (100 d) dietary exposure to ZEA at the dose of 25 or 50 µg/kg b.w. was found to produce profound regressive lesions in bitch reproductive system, e.g. granular cell degeneration, atrophy, uterine oedema or blood extravasations (6), which may lead to uterus infection (1).

Whereas a number of adverse effects in both laboratory and domestic animals have been assigned to treatment with ZEA, the molecular background of its toxicity remain relatively unknown. Recent evidences suggest that apart from its potential to alter oestrogen receptor (ER)-dependent gene transcription in ER-positive cells of the animal reproductive tract, ZEA is also able to interfere with other cellular pathways, such as steroid hormone biosynthesis (8, 12, 20). In a widely accepted biotransformation model (20), ZEA as a substrate for hydroxysteroid dehydrogenases (3α- and 3β-HSD) is being converted to α- and β-zearalenols (α- and β-ZOL), respectively, with species specific stereoisomeric ratio (10). Previous studies have shown that ZEA and/or its metabolites are capable to inhibit competitively 3α- and 3β-HSD, which may result in an accumulation of active components of other key steroidogenesis enzymes (e.g. P450 side chain cleavage; CYPscc), and in turn lead to the process impairment (12, 13). For example, in vitro exposure of mouse Leydig cells to ZEA or α-ZOL significantly suppressed testosterone secretion (previously induced by hCG), and the suppressive effect was correlated with down-regulation of 3β-HSD, CYPscc, and StAR mRNA levels (19). Furthermore, in the study of Tiemann et al. (17), the treatment of porcine granulosa cells with α- and β-ZOL reduced FSH- or forskolin-stimulated progesterone synthesis and mRNA levels of CYPscc and 3β-HSD in a dose-dependent manner, which was mentioned by Gajęcki et al. (5). The knowledge concerning the toxicity of ZEA in bitches is limited. Up-to-date information indicate that, no attempts were undertaken to estimate the influence of chronic intoxication with relatively low doses of ZEA (at NOAEL – no observable adverse effect level) on transcriptional regulation of key steroidogenic enzymes. In the presented study, sexually
immature beagle bitches were exposed per os to ZEA, and the effect of the treatment in terms of the expression of CYPsc and 3β-HSD mRNA in canine ovary was investigated.

Material and Methods

Animals and experimental conditions. The animals were handled in accordance with the regulations set forth by the Local Ethical Commission. Thirty immature beagle bitches, with an average age of 70 d and body weight of 8 kg, were obtained from the local breeders (registered at the Polish Kennel Club), and kept under standard conditions with free access to water. Clinically healthy individuals were divided into three equal experimental groups. Group A received orally ZEA (Sigma-Aldrich; Germany) at a dose of 50 µg/kg b.w. (100% NOAEL) (1), group B – ZEA at a dose of 75 µg/kg b.w. (150% NOAEL), and group C – placebo containing no ZEA administered per os (negative control group). After 42 d of the exposure, all bitches were anaesthetised and ovariectomised. Excised ovary samples were immediately immersed in the RNAlater™ solution (Sigma-Aldrich) according to the manufacturer’s recommendations, and stored at -20°C for further procedures.

Total RNA extraction and cDNA synthesis. Total RNA was extracted from the RNAlater™ preserved ovary tissues (approx. 20 mg per sample) using Total RNA Mini isolation kit (A&A Biotechnology; Poland), following DNase I treatment (Roche Diagnostics; Germany) to prevent genomic DNA contamination of the extracted samples. Total RNA quality and quantity were estimated using BioPhotometer (Eppendorf; Germany), and then the samples were used to synthesise cDNA with RevertAid™ First Strand cDNA Synthesis kit (Fermentas). The cDNA synthesis reaction mixture for each sample contained 1 µg of total RNA and 0.5 µg of oligo(dT)18 primers, and the reaction was performed according to the procedure recommended by the manufacturer. The synthesised first strand of cDNA was suspended in sterile DEPC-H2O and stored at -20°C until use.

Real-time PCR primers (Table 1) were either chosen from the literature or designed using Primer Express software (Applied Biosystems; USA). The assay was performed on ABI 7500 Real-time PCR System thermocycler (Applied Biosystems) in a singleplex mode, and all samples were analysed in duplicates. Each sample contained 10 µl of FastStart SYBR Green Master ROX mix (Roche Diagnostics), 3 pmol of each (forward and reverse) primer, 1 µl of cDNA as a template, and PCR-grade H2O to a final volume of 20 µl. The reaction was performed in standard thermal conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min. Negative water controls (NTCs) were included to rule out the possibility of cross-contamination. To verify the quality of PCR products, a melting curve analysis and agarose gel electrophoresis was performed after each run. In addition, representative PCR fragments produced by designed primer pair were sequenced (Genomed; Poland) and compared to sequences deposited in GenBank® (NCBI-NIH; USA).

Data analysis and statistics. Data obtained from the Real-time qPCR (threshold cycles; Cts) was used to compute mRNA expression ratios of CYPsc and 3β-HSD relative to RPS5 as an endogenous control. Calculations of the expression ratio were based on a gene individual PCR efficiency (E), and the threshold cycle difference (ΔCt) of an unknown sample versus a control (ΔCtcontrol-sample) according to the mathematical model: Ratio (ER) = [(Etarget)ΔCt target]/[(Ereference)ΔCt reference]+1 given by Pfaffl (14). The efficiencies for each gene were estimated by running reactions with dilution series of cDNA template with primer pairs used in the study, and the resulting Cts were plotted against cDNA concentrations to calculate respective slope values (data not shown). The corresponding efficiencies were calculated according to the equation: E = 10−1/slope (14) and were used as the basis for the gene expression calculations. Differences in gene expression between control and treated samples were assessed for statistical significance in group means by randomisation procedure (15).

Results

Real-time PCR efficiencies for each analysed primer set were high (>0.95) and the reaction specificity was confirmed with melting curve analysis and agarose gel electrophoresis. Amplicons for each primer pair were visualised as a single band at the expected length and the dissociation analysis yielded amplicon-specific melting temperatures (Table 1). Sequence analysis of the PCR products revealed high similarities to the canine mRNA sequences deposited in GenBank. No primer-dimer formations were generated during the applied 40 Real-time PCR amplification cycles.

Furthermore, the Ct values of the reference gene RPS5 measured in treated samples (experimental groups A and B) were not significantly different than those in the control group (P>0.05).

**Fig. 1.** CYPsc (grey bar) and 3β-HSD (white bar) mRNA expression in bitches ovaries after 42 d of oral exposure to ZEA with a daily dose of A) 50 or B) 75 µg/kg. Bars represent mean values of expression ratios (ER) with their respective standard errors of the mean (S.E.; n = 10), normalised by RPS5 as an endogenous reference, relative to control group C (ER = 1.00; dashed line). * P<0.05 between control and treated group.
increase in progesterone synthesis after 48 h, which was stimulated by appearance of specific substrates for the key steroidogenic proteins, and this process may be limited due to low activity of an enzyme (3, 18). For example, addition of pregnenolone to primary porcine granulosa cells led to an accumulation of 3β-HSD mRNA (ER=5.37; S.E. 1.83±14.89; P=0.016). Although insignificant, a slight up-regulation of CYPscc mRNA level (ER=2.44; S.E. 0.95±4.69; P=0.076) was also observed in the same experimental group of animals (150% NOAEL).

**Discussion**

To our knowledge, this is the first study documenting CYPscc and 3β-HSD mRNA expression changes in the ovary of sexually immature bitches after oral exposure to ZEA, which as a consequence may lead to imbalance of proteins involved in steroidogenesis. The balance is essential in several physiological functions, such as salt balance, metabolism of carbohydrates, or reproduction. In the first steps of steroid hormone biosynthesis, CYPscc converts cholesterol to pregnenolone, which is next transformed to progesterone by Δ5-Δ4 isomerases, 3β-HSDs (11). In ovarian follicle of sexually immature bitches, basal progesterone synthesis is limited due to low activity of the key steroidogenic proteins, and this process may be stimulated by appearance of specific substrates for the enzymes (3, 18). For example, addition of pregnenolone or forskolin to primary porcine granulosa cells led to an increase in progesterone synthesis after 48 h, which was accompanied by significant accumulation of 3β-HSD mRNA (17). It is known that intoxication with ZEA may similarly engage 3α- and 3β-HSD to convert the compound into major metabolites, α- and β-ZOL (20). If the apparent substrate dependent mRNA accumulation of 3β-HSD after treatment with pregnenolone or forskolin (17) is also triggered in response to stress molecules such as ZEA, it would explain mRNA accumulation of 3β-HSD in the ovaries of juvenile bitches after 42 d of treatment with the mycotoxin. Since our understanding of the molecular mechanisms that regulate steroidogenic gene expression still remains incomplete, we cannot exclude a possible involvement of an unknown transcription factor in the substrate-dependent gene expression of 3β-HSD.

In contrast, previous in vitro studies, concerning competitive inhibition of 3β-HSD, report the ability of ZEA and/or its metabolites (α-, β-ZOL) to suppress (previously stimulated) CYPscc and 3β-HSD mRNA levels in pig granulosa cells (16, 17) and mouse Leydig cells (19). These results, seemingly contradictory, may actually support the results of the present study, indicating that the 3β-HSD inhibition may serve as a negative feedback caused by the downstream products of ZEA biotransformation (i.e. α-and β-ZOL). If the above assumption is true, then the enzyme activity status and a dose of the toxicant will be crucial elements for either positive or negative effect on the gene transcription regulation. However, additional analyses, including measurement of ZEA, α-, and β-ZOL local concentrations and 3β-HSD protein level, are needed to provide an effective assessment of the presented hypothesis.

In conclusion, this is the first study documenting CYPscc and 3β-HSD mRNA levels in bitches ovaries after a long-term per os exposure to low doses of ZEA. The results show that while ZEA at the lower dose (100% NOAEL) did not cause effect, treatment with a higher dose (150% NOAEL) induced a significant increase in 3β-HSD mRNA levels in pig granulosa cells (16, 17) and mouse Leydig cells (19). These results, seemingly contradictory, may actually support the results of the present study, indicating that the 3β-HSD inhibition may serve as a negative feedback caused by the downstream products of ZEA biotransformation (i.e. α-and β-ZOL). If the above assumption is true, then the enzyme activity status and a dose of the toxicant will be crucial elements for either positive or negative effect on the gene transcription regulation. However, additional analyses, including measurement of ZEA, α-, and β-ZOL local concentrations and 3β-HSD protein level, are needed to provide an effective assessment of the presented hypothesis.

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**References**


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**Table 1**

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Sequence (5’→3’)</th>
<th>Amplicon length (bp)</th>
<th>Melting temp. (˚C)</th>
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<td></td>
<td>Reverse CCGACGGGTCTCTGGTGAT</td>
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<tr>
<td>3β-HSD</td>
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<td>78.6</td>
<td>(9)</td>
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<td>82.7</td>
<td>(2)</td>
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<td></td>
<td>Reverse CCTGATTTCACACGCGTAG</td>
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Figure 1 shows calculated expression ratios (ER) of CYPscc and 3β-HSD mRNA level in the ovaries of beagle bitches after 42 d of oral exposure to different doses of ZEA (50 and 75 µg/kg b.w.). The exposure to ZEA at a dose of 50 µg/kg b.w. (100% NOAEL) did not cause distinct effect on the examined gene expression. On the other hand, treatment with the higher dose of ZEA (75 µg/kg b.w.; 150% NOAEL) resulted in a moderate accumulation of 3β-HSD mRNA (ER=5.37; S.E. 1.83±14.89; P=0.016). Although insignificant, a slight up-regulation of CYPscc mRNA level (ER=2.44; S.E. 0.95±4.69; P=0.076) was also observed in the same experimental group of animals (150% NOAEL).


