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Individual and Combined Effects of Subchronic Exposure of Three Fusarium Toxins (Fumonisin B, Deoxynivalenol and Zearalenone) in Rabbit Bucks

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Abstract

Objective of the study was to determine reproductive toxicity of Fusarium toxins orally at three subchronic doses on adult Pannon White male rabbits. The four treatments were: control (C, toxin-free diet), F (5 mg/kg FB1), DZ (1 mg/kg DON+0.25 mg/kg ZEA), FDZ (5 mg/kg FB1+1 mg/kg DON+0.25 mg/kg ZEA) for 65 days (n=15/treatment). The doses were pre-determined according the EU limits in finished feed for young pig (in the absence of limits for rabbits' feed; based on the European Commission Recommendation 2006/576/EC and the European Commission Directive 2003/100/EC). The most pronounced effects of the toxins were exerted on the reproductive processes. The ratio of spermatozoa showing progressive forward motility decreased ($P<0.05$) from 80% to 67% in the FDZ group by day 60. Differences were found between the groups DZ (66.3% ± 23.7) and C (80.2% ± 11.2) in spermatozoa morphology. GnRH treated animals produced less testosterone in FDZ animals, compared to the other three groups ($P<0.05$). In the comet assay the individual fumonisin treatment resulted in significantly less 0 comets (intact cells), compared to all others. Based on the prevalence of score, lower ($P<0.0001$) damage was observed in FDZ group, as compared to F and DZ. Among the mycotoxins studied, additive or less than additive effect was found in case of spermatogenesis and sperm cell morphology, synergism in testosterone production, while FB1 acted antagonistically against DON+ZEA in comet assay. All mycotoxins provoked moderate lipid-peroxidation, based on the changes of glutathione concentration, glutathione peroxidase activity and formation of malondialdehyde and conjugated dienes and trienes, and exerted slight genotoxicity based on comet assay, FB1 being antagonistic towards DON+ZEA. In F, DZ and FDZ animals the intensity of spermatogenesis decreased by 43, 31 and 64%, respectively, which was reflected by lack of differentiated spermatozoa, thinning of the germinal epithelium, the appearance of multinuclear giant cells, indicative of the disturbance of meiosis and mitosis of the germinal epithelial cells and in some cases the lack of spermatogonia.

Keywords: Fumonisin; Deoxynivalenol; Zearalenon; Spermatogenesis; Testosterone production; Rabbit

Introduction

The worldwide contamination of foods and feeds with mycotoxins is a significant problem. Studies have shown extensive mycotoxin contamination in both developing and developed countries [1-3], as effect of global climate change [4].

The toxicity of mycotoxins differs depending on the kind of toxin, and in animals it is related to the species, the dose ingested, the duration of the exposure, and their gender and age. The main classes of Fusarium mycotoxins with respect to animal health and production are the non-oestrogenic trichothecenes such as deoxynivalenol (DON) and T-2 toxin (T-2), the myco-oestrogens including zearalenone (ZEA) and its zearalenol metabolites [5] and fumonisins.

Fumonisins (B1 and B2) are cancer-promoting metabolites of *Fusarium proliferatum* and *Fusarium verticillioides* that have a long-chain hydrocarbon unit (similar to that of sphingosine and sphinganine), which plays a role in their toxicity. Fumonisin B1 (FB1) is the most toxic and has been shown to promote tumour in rats and cause equine leukoencephalomalacia and porcine pulmonary oedema. FB, the most abundant of the numerous fumonisin analogues, was classified by the IARC as a Group 2B carcinogen (possibly carcinogenic in humans) [6].

Although DON is the least toxic type of trichothecene, it can cause significant harmful effects in animals and humans [7]. DON causes a broad variety of toxic effects in animals, and its toxicity is well recognized in mammals. The main effect at the cellular level is the inhibition of protein synthesis via binding to the ribosomal subunit [8]. Chronic oral exposure induces anorexia, decreased weight gain, reduction in feed conversion, gastrointestinal hemorrhaging, inflammation and immune system alterations.

ZEA competes with the naturally produced hormone estradiol-17β for binding sites (estradiol receptors) in various organs in the body of both genders. ZEA can obstruct normal steroid hormone (estradiol, testosterone, progesterone) synthesis in the ovaries and testicles of livestock.

ZEA is found, especially, as a contaminant in corn. ZEA, which is produced mainly by *F. graminearum* and *F. culmorum* and commonly

co-occurs with DON and its derivatives. It is among one of the most frequently encountered mycotoxins in grain from FHB (fusarium head blight)-diseased small-grain cereals throughout the Mediterranean countries. It may co-occur with DON in grains such as wheat, barley, oats and corn and fumonisins in corn [9]. Generally, DON is found in higher doses than ZEA when this occurs [10]. Most studies indicate that in artificial environment high moisture favours the production of both classes of mycotoxins, but the optimum temperatures for trichothecene and ZEA production in Fusarium-infected grain appears to be specific to the substrate, species and individual metabolites. The accumulated data revealed definite geographical differences in the level and frequency of DON, NIV and ZEA in wheat and barley. Streit et al. [11] reviewed mycotoxin co-occurrences in animal feed in Europe since 2004. Since Fusarium species are the most frequent fungal pathogens on field crops, it was not surprising that B-trichothecenes (DON), ZEA, and FBs were the major co-contaminants.

Reproductive efficiency is a very important economic factor in animal production. Exposure to several Fusarium mycotoxins have been linked to reproductive disorders as it was reviewed by Cortinovis et al [12]. The effect of these toxins on reproduction has been widely studied in female animals, while knowledge about their effect on reproduction processes in males is limited. As far as it is known they can affect spermatogenesis, and sperm quality, Leydig cell function (testosterone production), gonadotropin secretion, and fertility [13-18].

Humans and animals are generally exposed to multiple mycotoxins in parallel, as some mycotoxins typically co-occur in cereals. However, toxicological data, risk assessments are based on, are provided by studies in which only the individual effects of certain toxins are investigated. The simultaneous exposure of animals to more than one toxin is of concern and requires more study [19]. Synergistic effects may explain why animals sometimes respond negatively to mycotoxin levels much lower than those reported in scientific studies as able to cause mycotoxicoses.

In a survey conducted over a period of 4.5 years in countries of Southern Europe (Portugal, Spain, Italy, Greece and Cyprus) the Fusarium mycotoxins were found to be the major contaminants (fumonisins, type B trichothecenes and ZEA) of feed material and compounded feed samples [20]. There are several studies about mycotoxins' interaction (mainly in vitro), but only a few report the combined effects of three specific Fusarium mycotoxins which are more likely to co-occur; i.e. FB, DON and ZEA. Although there are studies about the three aforementioned mycotoxins very few focus on tertiary mixtures [21-24]. In rabbit bucks, ZEN impairs spermatogenesis and decreases libido, although only at high doses (117.3 mg/kg feed) [25]. Pregnant rabbit does fed on a DON-contaminated diet (0.3 and 0.6 mg/kg) showed marked bodyweight loss, but teratogenic effect was not proven [26]. FB1 was found to be nephron- and hepatotoxic in rabbits [27], and it has also been shown to negatively influence haematopoiesis by impairing bone marrow function [28]. Lung oedema was found only in a small number of rabbits fed a FB1-contaminated diet [29]. The teratogenic effect of FB1 was also described using an oral dose of 300 mg/d for 14 d [18] but a lack of embryotoxicity was found at lower (0.1, 0.5 or 1.00 mg/d) oral doses [30].

Grenier and Oswald [31] performed a meta-analysis of published raw data on mycotoxin interactions in vivo which varies according to the animal species, the dose of toxins, the length of exposure, but also the parameters measured; and classified the interaction into the following categories: synergistic, additive, less than additive, and antagonistic. The authors also differentiated between three types of synergistic effects and two types of antagonisms. Such characterization of mycotoxin interactions is helpful in experimental designs and interpretations of combined toxicity outcomes and should be included into further investigations on mycotoxin interactions.

The aim of this experiment was to study the dietary, low-dose of FB, DON and ZEA mycotoxins' individual and combined effects on breeding rabbit bucks, in particular with regards to reproductive toxicity.

Materials and Methods

Experimental animals, housing and feeding

The experimental animals were Pannon White rabbit breeding bucks (24 weeks of age, 4.0 ± 0.5 kg mean bodyweight, n=60). They were individually housed in wire mesh cages (42 × 50 cm) in a closed building, with 16 light h/day. Average temperature ranged from 16°C to 18°C and the farm had overpressure ventilation.

The animals received a commercial diet containing 10.3 MJ digestible energy/kg, 15.5% crude protein, 4.0% crude fat and 14.7% crude fibre for a total of 65 feeding days. The feedstuffs provided were available ad libitum, and the rabbits also had free access to drinking water.

Toxin production

Fusarium verticillioides (for FB1) and *Fusarium graminearum* (for DON and ZEA) (NRRL 20960 [MRC 826] and NRRL 5883, respectively) fungal culture (7 days old) was grown on 0.5 strength potato dextrose agar (PDA; Chemika-Biochemica, Basle, Switzerland). Agar discs (5 mm) were prepared with cork borer (Boekel Scientifica, Pennsylvania, USA), which were then stored at 10°C in darkness in test tubes containing sterile distilled water (10 discs/10 ml).

For toxin production, maize (40 g) was soaked in distilled water (40 ml) at room temperature for 1 hour in Erlenmeyer flasks (500 ml), closed with cotton wool plugs. This was followed by the addition of the inoculated agar discs (10 agar discs per flask) to the two-times autoclaved (20 min.) matrix. The cultures were then stored and incubated at 24°C (FB1), 28°C (DON) and 18°C (ZEA) for 3 weeks, respectively. The flasks were shaken twice every day during the first week of incubation. When the incubation time was complete the fungus-infected cereal was dried at room temperature and ground.

The homogenized fungal cultures contained FB, DON and ZEA at concentrations of 3300, 2010 and 1298 mg/kg, respectively. The European Commission has made recommendations (2006/576/EC) regarding the maximum level of several mycotoxins in complete diets (European Commission, 2006) and introduced regulations (2003/100/EC) regarding aflatoxins (European Commission, 2003). However, these only apply to certain cases, particularly with regard to rabbit feed. Thus in this study doses were pre-determined according the EU limits (based on the European Commission Recommendation 2006/576/EC and the European Commission Directive 2003/100/EC) in finished feed for young pig as the most sensitive animal towards these fusarium mycotoxins among livestock. Fungal cultures were mixed into the feed of experimental animals, based on the presented dose in Table 1.

Group	Mycotoxin concentration (mg/kg) of the feed	n
C (control)	0	15
F (FB1)	5	15
DZ (DON+ZEA)	1+0.25	15
FDZ (FB1+DON+ZEA)	5+1+0.25	15

Table 1: Experimental groups.

LC-MS analysis

LC-MS analysis was performed by a Shimadzu Prominence UFLC separation system equipped with a LC-MS-2020 single quadrupole (ultra-fast) liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) comprised a vacuum degasser, a binary pump (20AD), a column oven (CTO 20A) autosampler (SIL 20ACHT), and mass analyser (MS 2020) with both atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ESI) systems. Optimized mass spectra were obtained with an interface voltage of 4.5 kV, a detector voltage of 1.05 kV in negative mode, 1.25 kV in positive mode. Heat block temperature was 200°C and a desolvation gas temperature was 200°C. For nebulizing and drying gas, nitrogen was used (1.5 L/min and 15 L/min flow rate, respectively). Chromatographic separation was performed at 50°C and achieved on an RP-18 (2.1 × 100 mm, 2.6 μm, Kinetex^{TM} Phenomenex USA) stationary phase applying gradient elution 0.3 mL/min eluent total flow rate for mycotoxins and 0.4 mL/min for silymarin flavonoids, with A: 0.1% AcOH and B: 0.1% AcOH in methanol as eluent. With optimum method performance characteristics analytes were quantified using external calibration.

Rabbit-feed samples were milled and extracted using 1% AcOH containing 75:25=MeOH:H_2O (V/V) for F-2 CH_3CN:water 1:1 (V/V) for FB1 and water for DON and ZEA as extraction solvent. The extracts were shaken at room temperature for an hour then decanted and the supernatant was collected. 1 mL of clean water extract was applied to the immunoaffinity column (IAC; Vicam, DON test) which contains specific antibodies for DON for purification. The IAC was washed with 5 mL water, and DON was slowly eluted in 2 mL methanol. ZEA and FB1 were measured by dilute and shot method. Romer Mix 4 (containing trichotecenes+zearalenon at 10 mg/L) and Romer MIX 3 (containing FB1-2 at 50 mg/L) primary stock solution used as reference. 1 μL of each samples were analysed with a gradient: (0 min) 5% B, (3 min) 60% B, (8 min) 100% B, followed by a holding-time of 3 min at 100% eluent B and 3 min column re-equilibration at eluent A pumped at a flow rate of 0.3 mL/min. DON is detected as [M +AcO]– at m/z=355, ZEA at m/z=317 as [M-H]–, FB1 at m/z=722[M +H]+. The limit of detection (LOD) for FB, ZEA and DON was 3.0, 5.0 and 5.0 μg/kg, while the limit of quantification was (LOQ) 10, 1.0 and 2.0 μg/kg, respectively.

Experimental design

Bucks were divided into 4 experimental groups. One group of the experimental animals served as control, while into the feed of the other three groups fungal culture was mixed in a pre-defined concentration (Table 1).

The daily feed intake was registered by measuring back the left-over feed amount (in the first 3 weeks daily, and weekly thereafter), while bodyweight was recorded once every week.

The health status of the animals was observed throughout the experiment, morbidity and mortality was logged daily.

On days 30 and 60 blood and sperm was sampled (n=15/group), and a gonadotropin-releasing hormone (GnRH) test was performed (blood samplings related to GnRH test: n=6/group). Blood was sampled from the marginal ear vein, while sperm was collected after a training period into artificial vaginas and seminal plasma was separated with centrifugation.

At the end of the study (on day 65) animals were exsanguinated after stunning. The weight of liver, kidneys, testicles and spleen was measured and macroscopic changes were analyzed and recorded. After dissection (n=15/group) samples were taken for histopathological analysis; the testis, liver and kidney was fixed in 10% neutrally buffered formalin. For the analysis of antioxidant status 2-2 g samples were taken from the liver.

The experimental protocol was authorized by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under allowance number SOI/31/1679-11/2014.

Clinical chemical parameters

The plasma total protein (TP), albumin (ALB), globulin (GLOB), total cholesterol (tCHOL), triglyceride (TG), glucose (GLU), fructosamine (FA), creatinine (CREA) and bilirubin (BIL) concentrations, and the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALKP) were determined in a veterinary laboratory (Vet-med Laboratory, Budapest, Hungary), using Roche Hitachi 912 Chemistry Analyzer (Hitachi, Tokyo, Japan) with commercial diagnostic kits (Diagnosticum LTD., Budapest, Hungary).

Antioxidant status

For the determination of lipid peroxidation, the samples of blood plasma, red blood cell (1:9) hemolysate (RBCH) and liver were stored at -70°C until analysis. Lipid peroxidation was determined by the quantification of malondialdehyde (MDA) levels with 2-thiobarbituric acid method in blood plasma and RBCH [32] and liver homogenate [33], and determination of conjugated dienes (CD) and trienes (CT) according to the AOAC [34] method in the liver. Among the components of the antioxidant system some parameters of the glutathione redox system was determined in blood plasma, RBCH and liver. The amount of reduced glutathione (GSH) measured by the method of Sedlak and Lindsay [35] and the activity of glutathione peroxidase (GPx) according to Lawrence and Burk [36].

GnRH test and determination of testosterone concentration

Experimental and control bucks were treated i.m. with 0.2 ml GnRH analogue (Receptal; Intervet, Boxmeer, The Netherlands) for the analysis of the toxin effect on the Leydig cell function. The levels of testosterone hormone were determined from blood samples taken just prior to GnRH analogue injection (0 min) and thereafter in the subsequent 2 hours in every 25 minutes (a total of 6 blood samplings).

The testosterone concentration was determined with a direct ^{3}H-radioimmunoassay method [37] adopted and validated for rodents' (chinchilla rabbit and Angora rabbit) plasma as described previously [38].

Spermatology

Spermatological analyses covered the following parameters: pH, sperm cell concentration (improved Neubauer cell counting chamber), motility, morphology (native and stained) and acrosomal integrity (Spermac^{TM} staining, Beernem, Belgium) of the spermatozoa. Motility was evaluated with a computer assisted sperm analyzer (Medealab^{TM} CASA System, Erlangen, Germany). Moreover, vital test, hyposmotic and peroxidase tests were carried out. A minimum number of 200 spermatozoa were examined for morphology and 500 for motility evaluation [39].

Comet assay

For comet assay sperm was sampled on day 60 of the experiment (n=15/group). The method was adapted from human spermium examination protocols [40,41], with the following modifications. The semen was washed three times in PBS and re-suspended in PBS to a final of 10^{6} cell/ml number. Onto the microscope slides pre-coated with 1% NMP agarose 10 µl cell suspensions and 75 µl 1% LMP agarose were loaded. The decondensation was performed in two steps, first the slides were soaked for 1 hour at 4°C in a lysis buffer with dithiothreitol, and second the slides were soaked for 1 hour at 37°C in a lysis buffer with proteinase K. After lysis the slides were washed in sterile redistilled water to eliminate the salt adhered to the gel. Electrophoresis was performed at 300 mA and 25 V for 30 minutes at 4°C. After washing and drying the slides were stained with ethidium bromide. All chemicals used in this study were obtained from Sigma-Aldrich Ltd. (Budapest, Hungary). Specialized chemicals used were: Histopaque-1077 and RPMI-1640 medium (Sigma-Aldrich Ltd., Budapest, Hungary).

The fluorescence images were generated using an Alpha-Optika B-600TiFL fluorescence microscope (Optika Microscopes, Bergamo, Italy). Scoring was carried out according to Singh et al. [42] and Collins et al. [43], in which comets are classified into scores of '0', '1', '2', '3' and '4' according to DNA damage and head/tail migration. Each single comet was scored visually and assigned into an arbitrary unit from 0 to 4, depending on the relative intensity of DNA fluorescence in the tail; 800 cells/group were counted.

Histopathological analysis

After registering the macroscopic pathological signs on the internal and external organs, testicles, liver, kidneys and spleen were stored in 10% neutrally buffered formalin and were embedded into paraffin. For light microscopic analysis microtome slides of 5 micrometer were prepared and stained with hematoxylin-eosin.

The histopathological analysis was performed according to the Act/2011 (03.30) of the Hungarian Ministry of Agriculture and Rural Development and was in accordance with the ethical guidelines of the OECD Good Laboratory Practice for Chemicals [44].

Statistical analyses

Statistical analyses were performed using IBM SPSS 20.0 [45] software. Data processing and the mathematical-statistical calculations were performed using the compare means (independent-samples-t-test, oneway ANOVA with Tukey post-hoc test), correlate and descriptive statistics modules. In case of comet assay crosstabs options were used for chi-square test.

Results

Feed consumption and body weight

The feed intake of the rabbit bucks was not different among the groups. No significant difference in body weight among groups was detected at any of the 12 time points (data not shown), average body weight of the groups was between 4252 and 4442 g by the end of the experiment.

Clinico-chemical parameters

No inter-group differences ($P \geq 0.05$) were found for ALB, TG, GLU, FA, CREA and BIL concentrations, and AST, ALT, GGT, LDH and ALKP activities.

		Day 30		Day 60	
		mean	SD	mean	SD
TP (g/L)	C	69.85^{b}	5.85	62.16^{a}	3.02
	F	68.57^{b}	1.72	64.73^{ab}	3.97
	DZ	61.33^{a}	4.28	65.81^{ab}	7.34
	FDZ	69.57^{b}	1.92	67.74^{b}	3.40
GLOB (g/L)	C	28.65^{b}	3.54	22.38^{a}	2.23
	F	27.13^{b}	1.61	22.96^{a}	2.09
	DZ	20.23^{a}	3.39	24.10^{ab}	5.95
	FDZ	27.98^{b}	1.63	26.67^{b}	3.12
tCHOL (mmol/L)	C	1.45^{ab}	0.12	1.51^{b}	0.35
	F	1.62^{b}	0.19	1.21^{a}	0.09
	DZ	1.33^{a}	0.16	1.29^{a}	0.14
	FDZ	1.58^{ab}	0.17	1.44^{ab}	0.14

^{a,b}numbers with different superscripts indicate significant differences ($P \leq 0.05$) between groups (C, F, DZ and FDZ).

Table 2: Clinico-chemical parameters of rabbits on day 30 and 60.

Differences in TP, GLOB and tCHOL at the two sampling dates (day 30 and 60) are summarized in Table 2. In spite of the slight significant differences between treatments all data were within the reference ranges, which are 54-75 g/L for TP, 15-27 g/L for GLOB and 0.3-3.00 mmol/L for tCHOL [46]. Only GLOB concentration exceeded the upper limit of 27 g/L in the C and FDZ groups on day 30 (29 and 28 g/L, respectively).

Antioxidant status

On day 30 only MDA concentration in the RBCH showed significant difference between treatments F (35.7 ± 4.5 mmol/l) and DZ (34.2 ± 1.6 mmol/l), as compared to C (43.4 ± 3.5 mmol/l) and

FDZ (43.7 ± 7.6 mmol/l), but other antioxidant parameters did not change significantly (data not shown).

Parameters showing significant treatment effect on day 60 are summarised in Table 3. At day 60, DZ treatment resulted in significantly increased GPx activity in the red blood cells and MDA formation both in RBCH and plasma, while less GSH concentration in the blood plasma. As a result of peroxidation of dienoic and trienoic fatty acids, the concentration of conjugated dienes and trienes increased as a result of the DZ exposure, as compared to the combined effect of three mycotoxins (FDZ).

Parameter	Groups			
	C	F	DZ	FDZ
GPx-RBCH[1]	2.7 ± 1.0[a]	3.5 ± 1.2[a]	4.9 ± 1.1[b]	3.1 ± 0.8[a]
MDA-RBCH[2]	30.3 ± 3.0[ab]	28.3 ± 3.2[a]	33.9 ± 7.5[b]	28.8 ± 3.2[a]
GSH-plasma[3]	2.2 ± 0.2[a]	2.2 ± 0.5[a]	2.0 ± 0.2[a]	2.6 ± 0.5[b]
MDA-plasma[2]	18.9 ± 2.8[a]	18.7 ± 3.0[a]	24.5 ± 8.0[b]	16.8 ± 2.9[a]
CD liver[4]	0.51 ± 0.03[ab]	0.51 ± 0.02[ab]	0.53 ± 0.05[b]	0.48 ± 0.05[a]
CT liver[4]	0.19 ± 0.01[a]	0.20 ± 0.01[ab]	0.21 ± 0.04[b]	0.18 ± 0.01[a]

[1]U/g protein,[2]µmol/ml,[3]µmol/g protein,[4]Abs 232 nm

[a,b]numbers with different superscripts indicate significant differences (P ≤ 0.05) between groups (C, F, DZ and FDZ)

Table 3: Antioxidant parameters measured on day 60.

Testosterone concentration

The testosterone concentration was significantly different due to mycotoxin exposure only at day 60 at the sampling minutes of 75, 90 and 115 (Figure 1). Figure 1 clearly demonstrates that Leydig cells of the animals intoxicated with the three mycotoxins in combination (FDZ) for a total of 60 days synthesize significantly less testosterone as a response to exogenous GnRH. In the F group blood testosterone level was slightly lowered, while the two toxin combination (DZ) induced a more expressed decline in the concentration, although the differences were not statistically significant.

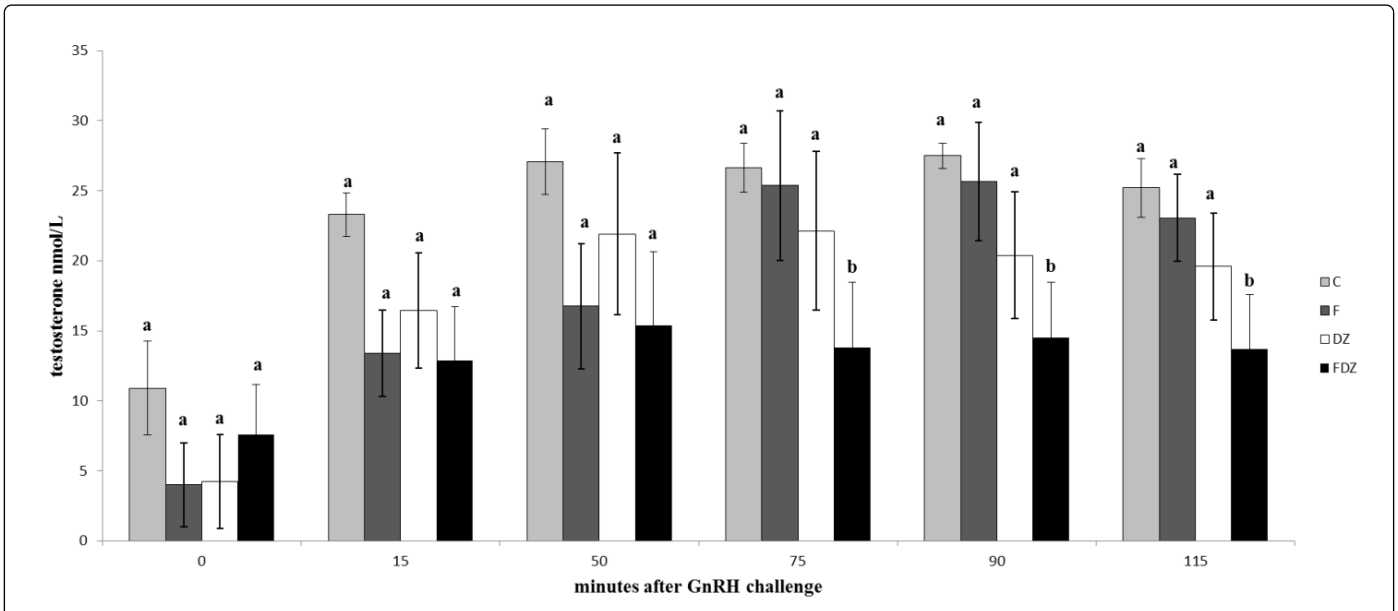

Figure 1: Gonadotropin-releasing hormone (GnRH; mean and standard deviation) induced testosterone production at day 60. [a,b]numbers with different superscripts indicate significant differences (P ≤ 0.05) between groups (C, F, DZ and FDZ).

Spermatological analysis

No effect of toxin treatment was detected on semen pH (ranging from 6.4 to 8.2 in each group), quantity of the semen (on average 1 ml in each group) and concentration of spermatozoa (ranging from 2.4 to 2.6 × 10^7/ml). Comparing the sperm motility between groups, significant effect could also not be proven. The ratio of spermatozoa showing progressive forward motility was around 80% at the

beginning of the experiment and decreased from 80 ± 1.7% to 67 ± 4% in the semen of FDZ animals showing significant difference when compared to the other three treatment groups. Between the normal morphology of the spermatozoa of the DZ and C animals, statistically significant differences were found (Table 4).

Group	Spermatozoa with normal morphology
C	80.2 ± 11.2^{b}
F	76.0 ± 9.0^{ab}
DZ	66.3 ± 23.7^{a}
FDZ	68.9 ± 14.1^{ab}
^{a,b}different superscripts in the column indicate significant between-group differences (P ≤0.05)	

Table 4: The ratio of spermatozoa with normal morphology in the semen after 60 days of mycotoxin exposure (%, mean ± SD).

The most frequent morphological abnormalities were: abnormality of the tail, retention of cytoplasmic drop, absence of the acrosome and altered head. A cell was considered altered if at least one defect was present.

Comet assay

All treatments caused DNA damage and 98.6, 91.6 and 91.8% of the treated cells could be categorized as having 1 to 3 scores in the F, DZ and FDZ group, respectively (Figure 2). Cells with 0 values showed the highest prevalence in group C, while cells with the slightest damage (score 1) were dominant in all toxin treated groups. FB1 resulted significantly less 0 comets (intact cells) compared to the other treatments.

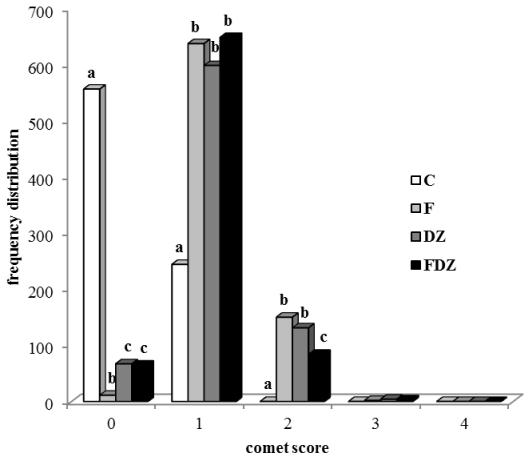

Figure 2: Number of cells with the respective comet values (values of comet scores: 0 to 4). ^{a,b}columns with different superscripts indicate significant differences (P<0.0001).

Cells, with score 1 (Figure 3a) occurred in nearly similar proportions in case of the three toxin treatments. The comet value 2 (Figure 3b) showed the highest prevalence in samples of F and DZ animals, FDZ treatment resulted in significantly less cells of this type. The number of cells with score 3 (Figure 3c) occurred only in 0.6% of the categorized cells. No cells were found with a comet assay score of 4 in the experiment.

Figure 3: Comet assay (DNA damage) scoring: images of comets from bucks' spermatozoa stained with EtBr. Classes 0 (a), 1 (b) and 2 (c) were used for visual scoring.

Histopathology

No sign of mycotoxicosis was detected. In animals consumed FB1, DZ and FDZ contaminated diet the intensity of spermatogensis decreased by 43, 31 and 64%, respectively (Table 5), which was reflected by the lack of differentiated spermatozoa, thinning of the germinal epithelium, the appearance of multinuclear giant cells indicative of the disturbance of meiosis and mitosis of the germinal epithelial cells and in some cases the lack of spermatogonia.

These histological findings were observed to different severities in the seminiferous tubules. In case of slight alteration (grade 1) disturbance of sperm cell formation was observed in 20-30% of the seminiferous tubules, while in case of grade 2 and 3, 30-70 or 100 % of the tubules, respectively, showed decreased spermatogenesis. Decreased spermatogenesis was manifested in lack of well differentiated sperm cells and less primary, secondary spermatocytes and spermatids, and thinning of the seminiferous epithelium as a consequence (grade 1). In case of intermediate severity (grade 2) these alterations were observed in higher ratio of the tubules, the disturbance of meiosis and mitosis was indicated by the appearance of multinuclear cells. Cases, when all tubules were attacked and moreover, in some tubules even the initial spermiogenetic cells, spermatogonia were also absent, were classified as marked damage (grade 3) (Figures 4 and 5).

Group	NO	Number of animals showing alteration				
		1	2	3	All together	%
C	15	0	0	0	0	0
F	8	6	0	0	6	43
DZ	9	4	0	0	4	31
FDZ	5	7	1	1	9	64
NO=number of animals without alteration, 1=slight/small area/low occurrence frequency, 2=intermediate severity/intermediate area/intermediate occurrence frequency, 3=marked/extensive/high occurrence frequency						

Table 5: Decreased intensity of spermatogenesis as revealed by the histopathology of the testicles.

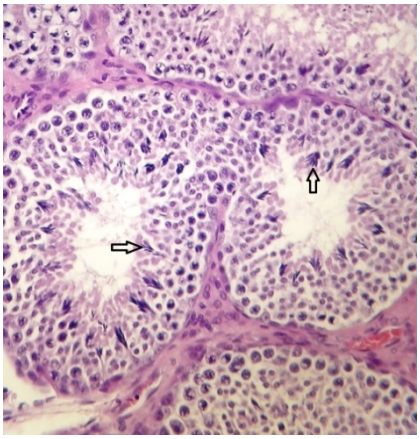

Figure 4: Active spermatogenesis in a healthy control animal's seminiferous tubules. The cells at different differentiation levels in the epithelium germinativum (spermatogonia, stage I and II spermatocytes, spermatides and mature spermia (↑) are as well visible. Haematoxylin-eosin staining, 400X.

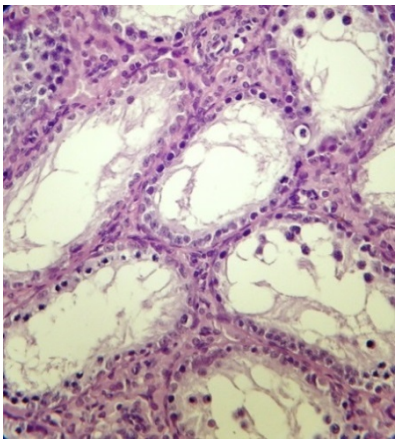

Figure 5: A part of the seminiferous tubule after FDZ treatment. The marked lack of germinal epithelialcells is visible. Haematoxylin-eosin staining, 400X.

In the testicles of the toxin treated animals the cytomorphology and the proportion of the Leydig interstitial cells was not significantly different from the controls. In the Malpighian bodies of the spleen of every DZ and FDZ animal, slight lymphocyte depletion (slight thinning of the T and B dependent zones of the Malpighian tubules) was observed. However, the cytomorphology of lymphoblast and lymphocyte cells was not different from the control. In the livers and kidneys no detectable alteration attributable to mycotoxins was found.

Organ masses

Among all organs which were measured for weight (testicles, liver, kidneys, spleen) only the weight of the spleen was significantly different between groups. It was the most (1.84 ± 0.49^{b}) in DZ animals, and least (1.46 ± 0.38^{a}) in C, while 1.65 ± 0.30^{ab} and 1.65 ± 0.37^{ab} in F and FDZ, respectively. However the relative organ mass values (data not shown) of the different groups were not significantly different.

Discussion

The present study intended to determine the negative effect of a combined, 65-day oral Fusarium mycotoxin administration on the reproductive traits of 24-week old breeding Pannon White rabbit bucks. The low toxin exposure levels were chosen taking the limit values given by the 2006/576/EC recommendation into consideration. The in feed concentrations of FB, DON and ZEA (5, 1 and 0.25 mg/kg feed, respectively) revealed 169-193 µg FB1/kg BW, 33.7-38.7 µg DON/kg BW, and 8.5-9.7 µg ZEA/kg bw exposure. Results indicate that a prolonged low-dose mycotoxin exposure may adversely affect male reproduction as it has been previously demonstrated in case of T-2 toxin. The combined toxin effect indicates highly complex interactions among the mycotoxins, appearing differently in the divergent physiological processes. Among the mycotoxins studied here additive, synergistic, as well as antagonistic effects were detected (Table 6).

Parameter examined	Type of interaction
feed intake	antagonism (not significant)
lipid peroxidation (MDA, GPx, CD, CT)	antagonism
testosterone production	synergism
spermatogenesis by histology	additive
sperm cells with normal morphology	less than additive
weight of the spleen	antagonism
genotoxicity	antagonism

Table 6: Summary of the interactions between FB, ZEA and DON experienced in the experiment (classified according to Grenier and Oswald, 2011).

Feed consumption and body weight

The only observation to mention concerning feed consumption and body weight (BW) was that the mean feed intake (FI) of DZ and FDZ animals was somewhat lower, as compared to F and C (significance was not proven). This result may mostly be attributed to DON, since this is the most well-known characteristic effect of trichothecene mycotoxins [47]. In the study of Hewit et al. [48] using naturally contaminated (4.2 mg/kg DON) corn for Fusarium mycotoxin exposure in growing rabbits, no feed refusal was observed and the toxin treated animals had slightly increased BW gain, suggesting that rabbits are less sensitive to this mycotoxin. Deep discussion of a mere tendency is void, but observing data at weeks 4 and 5 again indicate the "likewise positive effect" of low-dose FB, since the lowest FI was found for DZ, but FDZ and F were less suppressed. This is supported by the result on non-human primates of Gelderblom et al. [49], where long-term feeding of FB1 alone had no negative effect on FI and BW, even at higher doses. In that study apparent no-effect threshold in terms of kidney and liver damage was estimated at 8.21 and 13.25 mg total fumonisin/kg diet, being about the double exposure, as compared to the present study. Also no effect on final live weight and weight gain was observed when male rabbits were fed diets containing 12.3 and 24.5 mg/kg FB1 for 5 weeks [50]. Summarized, our study indicates that

DON provides its characteristic feed-intake lowering effect in a slight, but insignificant rate at the dose according to Commission Recommendation (2006/576/EC), while fumonisin shows no determinative effect on BW and FI at these concentrations, in breeding rabbit bucks.

Clinical chemical parameters and antioxidant status

The health status of the animals can be monitored by regular determination of certain clinical chemical parameters. Despite that the liver and the kidney is considered to be the primary target organs of many mycotoxins (like trichothecenes and FB1) in all species examined, gross hepatic lesions and disorder was not induced by any of the treatments, as underscored by the unaltered ALT, AST, GGT and CREA values, the identical liver and kidney mass values and the lack of histopathological alterations in all groups. These organs were likewise tolerant towards the exposure of low dose of these mycotoxins, without providing degenerative processes. Sprando et al. [14] described an analogous observation, namely few lesions of various tissues in control and treated animals, either incidental or related to experimental techniques (gavage) and unrelated to the test substance. The effects of low (10 mg/kgBW) and high (100 mg/kgBW) oral doses of purified ZEN were studied by Čonková et al [47] in rabbits. Low ZEN doses resulted in a significant increase in ALP activity, while high ZEN doses showed significant increases of AST, ALT, AP, GGT, and LDH activity, indicating possible liver toxicity due to the chronic effects of the toxin.

The alteration found in the TP was attributed to the concentration changes of globulin, since albumin concentration was constant. According to Kim et al. [51], DON stress lowers immunoglobulin expression in mice, but ZEA was found to have no influence on it. This could be the reason of the slight temporary decrease in GLOB and TP of DZ animals at day 30. The results at day 60 are difficult to explain, because difference between groups was rather attributable to the lowered GLOB level in control animals than toxin treatments. However fumonisin treatment decreased GLOB concentration at day 60, compared to day 30. Results of the FDZ group on day 60 are in accordance with those of Tessari et al. [52], describing that FB1 (added to an aflatoxin B1 containing diet) has primary additive effect on the immunological response in birds. It is interesting to highlight that FB1 alone and in higher doses (8 mg/kg feed) had a sex-dependent immunosuppressive effect, male pigs being more prone [53]. The FDZ treatment evokes the question, whether the low dose of FB1 or the interaction between these toxins augmented the immune response. Some kind of anomaly in the immune system was supported by histology, in that in both DZ and FDZ animals, slight lymphocyte depletion (slight thinning of the T and B dependent zones of the Malpighian tubules) was observed in the spleen, without any change in the cytomorphology of lymphoblasts and lymphocytes. In the experiment Sprando et al. [14] similar finding was described when rats orally exposed with 5 mg/kg DON.

The pathophysiological significance of the changes in tCHOL level, which was around 5-20%, is questionable, taking the broad physiological ranges (0.3-3.00 mmol/L) into consideration.

The increased GPx activity in the red blood cells of DZ animals by day 60 reflects more lipid hydroperoxide production, as the role of the enzyme is to reduce lipid hydroperoxides to their corresponding alcohols and this way to reduce free hydrogen peroxide to water. This is supported by the highest MDA concentrations both in the red blood cells and the blood plasma, referring to augmented in vivo lipid peroxidation [54], and also by the decreased GSH, as co-substrate of GPx, level in plasma. One of the mechanisms by which DON exerts its toxicity is inducing oxidative stress within the cells [55]. ZEA is also known to induce generation of reactive oxygen species (ROS) and consequently lipidperoxidation, while fumonisin was characterised as moderate oxidant mycotoxin [56]. Our results show general agreement with those of Minervini et al. [57], reporting that in vitro FB1 dose at the level of the EC Regulation is not inducing oxidative stress in intestinal cells. On the other hand FB1 has proven to induce ROS production in broiler chicks, but only at extreme high (100 mg/kg feed) dose [58]. Our findings suggest that FB1 in low dose may act antagonistically towards Z and D, which has not been reported yet. Though there is no literature on the mitigating property of FB1 on lipid peroxidation. We thus summarize DZ combination was the most harmful in terms of initial (CD and CT) and terminal (MDA) phases of lipid peroxidation after long-term exposure (60 days); meanwhile FB1 at the EC Regulation level did not cause significant oxidative stress, even more relieved those effects triggered by ZEA and DON.

Gn-RH induced testosterone production

The testosterone concentration was not significantly influenced by one (F) and two toxins (DZ), but the FDZ treatment dramatically lowered its synthesis and/or release from the Leydig cells by the end of the sampling intervals (minutes 75, 90 and 115), suggesting synergistic effect. As far as the authors are aware, there exists only a single study on male reproduction traits as affected by orally administered DON, which was performed in rats [14]. In this study 0.5, 1, 2.5 and 5 mg/kg DON was applied, and the authors found a dose-dependent down-regulation of testosterone synthesis after 28 days of exposure. Trichothecene toxins may decrease the testosterone production of the Leydig cells by inhibiting early steps of the steroidogenic pathway, i.e. the conversion of pregnenolone to progesterone [25], as it was also observed in our previous study in case of T-2 exposure [38]. These Fusarium toxins may act indirectly as well, on the pituitary gland, or affect Sertoli cell inhibin production, as shown in case of DON [14]. In present study a direct effect of the mycotoxin on Leydig cells was not supported by the histopathological findings.

In fact not DON, but zearalenone is the most potent factor, directly inhibiting testosterone biosynthesis in the Leydig cells, adding that this inhibition is concentration- and time-dependent, happening via changing the nuclear estrogen receptor signaling [59], however the exact molecular mechanism is not known yet. For FB1 direct toxicological studies are lacking, but according to Lu et al. [60], elevated ceramide levels are directly apoptotic towards Leydig cells, which is inhibited by FB, a ceramide-synthase blocking substance. Thus FB1 had no deleterious effect on the rat testicular Leydig cell function in vitro. In summary the main, testosterone synthesis blocking effect in our study was primarily attributed to ZEA. Some kind of synergistic effect occurred between FB1 and DON+ZEA. Taking the common cellular modes of actions of these toxins into consideration, this might be explained with respect to the DNA-synthesis inhibiting effect. FB1 affected the DNA synthesis together with ZEA in a more than additive, rather synergistic way in a DNA inhibition assay, although this effect was not fully confirmed when it was further analysed in a second stage [61].

Spermatology, histopathology and comet assay

Toxin effect and interaction between the Fusarium toxins applied, manifested in lower sperm motility (FDZ), less spermatozoa with

normal morphology (DZ), additive effect in the decreased intensity of spermatogenesis as shown by the histology of the testes, while antagonism concerning genotoxicity measured by comet assay.

Concerning the individual effects of these mycotoxins on sperm motility, only a few data are available. FB1 fed with male rabbits at 7.5 and 10.0 mg/kg dietary levels decreased sperm motility by approximately 18%, the possible explanation was the inhibition of cyclic 3'5'AMP activity and calcium ion transport, or, the decreased formation of the acidic epididymidal glycoprotein synthesis required to maintain motility [50]. Changes, after increasing oral exposure of DON were not consequent, and considered to be random [14], while in an in vitro study no significant effect of DON on motility was found using rabbit spermatozoa (Medvedová et al., 2012). Boeira et al. [62] reported compromised spermatozoa motility after feeding mice with ZEA, while 12 mg daily ZEA exposure for 8 weeks did not alter sperm motility in adult rams [63]. Further data from in vitro studies support no effect of ZEA on boar spermatozoa [64], while on the contrary Rajkovic et al. [65] and Tsakmakidis et al. [66] described impairment of progressive motility. Interestingly in an in vitro study with stallion sperm ZEA caused hyperactivation in motility [67].

As the cellular level effects by which the above described mycotoxins may adversely influence motility are not well described yet, the combined effect is also difficult to explain. Decreased sperm motility caused by the exposure of the three mycotoxins (FDZ) by day 60 might be attributable also to the decreased testosterone concentration as a result of synergistic effect between FB, DON and ZEA observed also by day 60. The male reproductive organs are strongly androgen-dependent in respect of structure, as well as function. Testosterone supports spermatogenesis, sperm maturation, seminal plasma production and sexual functions [68]. So the decreased testosterone secretion may give an explanation also for the more morphologically abnormal cells, and the decreased intensity of spermatogenesis shown by histology. However, direct effect of the particular toxins is also presumable. Interestingly FB1 had only a slight adverse effect on the cell morphology and spermatogenesis. The characteristic toxic effect of FB1 is the inhibition of the sphingolipid synthesis, thus causing cellular ceramide depletion and elevation of shinganine, which is cytotoxic, and has growth inhibitory and pre-apoptotic effects [69]. This results in disturbances of cell growth, differentiation and morphology. In rabbits orally administered FB1 caused similar morphological and structural abnormalities in sperm morphology and spermatogenesis, and 7.5 mg/kg in the diet for FB1 as LOAEL (lowest observed adverse effect level) was suggested [50]. In our study even lower dose, 5 mg/kg had exerted adverse effect; however sensitivity to toxic substance is influenced by several factors. Compared to F group, DZ was similarly compromised. In case F and DZ, disturbance of sperm cell formation was observed only in 20-30% of the seminiferous tubules, however the combined treatment of DON and ZEA resulted in more cells of abnormal morphology. After exposure of the three toxin (FDZ) in 1 animal 30-70, while in another 100% of the seminiferous tubules were concerned, indicating additive effect. The effect of ZEA on spermatogenesis is more thoroughly studied compared to FB1 and DON. In orally exposed mice Zatecka et al. [70] detected decreased sperm concentration, and increased number of morphologically abnormal and also apoptotic spermatozoa. No pathological patterns in the seminiferous tubules were observed and the spermatogenesis was not interrupted, only the number of spermatogonia and spermatocytes were lower (not significantly) in exposed animals (25 μg/kg BW). Kim et al. [71] highlighted that apoptosis is the main effect by which ZEA causes germ cell depletion and atrophy of the testes. Germ cell apoptosis and thus impaired spermatogenesis was described also by Cho et al [72].

The in vitro exposure to ZEA induced cytotoxicity in boar sperm cells [64]. DON in 2.5 and 5 mg/kg concentration caused germ cell degeneration, decreased sperm cell release and abnormal cell development [14]. As the authors reported, DON may inhibit protein, RNA and DNA synthesis, and be cytotoxic to certain cells, which could cause the harmful effect in the most sensitive pre-leptotene spermatocytes.

Genotoxicity of Fusarium mycotoxins on sperm cells has been reported previously in case of FB1 [17] and ZEA [17,64,66,67], using sperm chromatin structure assay (SCSA), while no data on genotoxicity of DON related to sperm cells are available. A recent study showed that DON decreased cell viability, caused damage to the membrane, the chromosomes and the DNA in human lymphocytes, and potentially induced genotoxicity by the depletion of the antioxidant capacity. On the other hand according to Golli-Bennour and Bacha [56] DON is a non-oxidant mycotoxin, and its genotoxicity is the result of a direct effect on DNA fragmentation and caspade-dependent apoptosis.

In case of FB, Minervini et al. [17] found high degree of individual variability, when chromatin structure stability was checked in vitro, using equine spermatozoa. Interestingly no increase in ROS production was observed even in those cases when genotoxicity was detected, so the damage was attributed to mitochondrial dysfunction related to the altered ceramide metabolism. This mode of action might influence motility as well, knowing that spermatozoa are used as biosensors when testing mitochondrial toxicity, because of the strong influence of mitochondrial function on motility [17].

When we examined the single and combined genotoxic effect of FB1 and DON+ZEA on rabbit sperm cells, an antagonistic effect was observed, highly in accordance with the antioxidant parameters of the same experiment. It is also worth to mention, that none of the toxins had strong genotoxic effect as shown by the total lack of highly damaged cells (score 4). To our knowledge this is the first study analyzing the DNA damage on sperm cells by comet assay in orally mycotoxin exposed animals. There is only one in vitro study using sperm cells for comet assay in mycotoxin genotoxicity studies [73-75], in which human spermatozoa and lymphocytes were assessed for their sensitivity to the genotoxic effect of DON. It was described that spermatozoa are more sensitive towards DON (in terms of DNA damage) as compared to lymphocytes. The reason may be that spermatozoa are a highly specialized cell types with no cytoplasm and extremely condensed chromatin, which seems to be prone towards toxic stimuli. Thus the moderate genotoxic effect of DON has been proven and spermatozoa comet assay as a rapid test for genotoxicity recommended.

Conclusions

In this study three orally administered fusariotoxins (FB, DON and ZEA) were tested in adult male rabbits, focusing primarily on their reproduction endpoints. The dietary levels were relatively low according to the EU limits in finished feed for young pig, in the absence of limits for rabbits' feed (based on the European Commission Recommendation 2006/576/EC and the European Commission Directive 2003/100/EC). Results indicate that a prolonged low-dose mycotoxin exposure may adversely affect male reproduction. Reproductive processes show higher sensitivity to toxic effects, shown

by the different parameters examined. Among the mycotoxins studied additive or less than additive effect was found in case of spermatogenesis and sperm cell morphology, synergism in testosterone production, while FB1 acted antagonistic against DON +ZEA on feed intake, lipid-peroxidation, and genotoxicity. All mycotoxins provoked moderate lipid-peroxidation and exerted slight genotoxicity.

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