Effect of subchronic oral exposure to zearalenone on the reproductive system of rabbit bucks

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OBJECTIVE
To determine the effect of subchronic oral exposure to zearalenone (ZEA) at a daily dose of 50 µg of ZEA/kg of body weight (an environmentally relevant concentration) on the reproductive system of rabbit bucks.

ANIMALS
8 healthy sexually mature New Zealand White rabbits.

PROCEDURES
During the experimental period (March to June), each rabbit underwent a 7-week control protocol and then a 7-week treatment protocol. Water (0.5 mL) or ZEA solution (50 µg/kg [0.5 mL]) was administered orally once daily during the control and treatment period, respectively; ejaculates were collected weekly. Studied end points included semen quality variables (spermatozoa kinetics, morphology, viability, and DNA fragmentation), serum testosterone concentration, and results of histologic examination of the testes and epididymides following euthanasia at the end of the experimental period.

RESULTS
Treatment with ZEA solution resulted in significant increases in spermatozoa beat-cross frequency, in the percentages of spermatozoa with head and midpiece abnormalities, and in the percentages of DNA-fragmented spermatozoa, compared with effects of the control treatment. Serum testosterone concentration, other spermatozoa velocity variables, and percentages of progressive and total motility, rapidly or slowly moving spermatozoa, and live spermatozoa did not differ significantly between the 2 periods. Histologic examination revealed no patterns of abnormal findings in the testes and epididymides.

CONCLUSIONS AND CLINICAL RELEVANCE
Oral treatment with ZEA solution at an environmentally relevant concentration caused minor interference with rabbit bucks’ sperm quality. Although mostly considered mild, the sperm quality changes warrant further investigation in terms of fertilizing capacity impairment. (Am J Vet Res 2018;79:674–681)

Zearalenone is a nonsteroidal estrogenic mycotoxin produced by Fusarium molds (mainly Fusarium graminearum and Fusarium culmorum) with worldwide occurrence in cereals and grains. Levels of ZEA contamination in feeds vary depending on the climatic zone and harvesting or storage conditions. On a worldwide scale, ZEA has been detected at concentrations ranging from 0.001 to 600 mg/kg of feed.1 Zearalenone, as its major metabolites α- and β-zearalenol, exerts its toxic effects mostly through competitive binding to estrogen receptors in various organs and by interference with steroid metabolism.2 Therefore, ZEA is considered primarily a reproductive toxicant, although genotoxic, hematotoxic, hepatotoxic, nephrotoxic, and immunotoxic properties have also been ascribed to it.3 Metabolic pathways elicited following ZEA exposure vary depending on the species affected; swine are currently considered the farm animal species that is most susceptible to ZEA toxicosis, among which females are more commonly affected.4

There has been considerable discussion in the veterinary medical literature about the reproductive disorders that develop in farm animals as a result of ZEA exposure, even after exposure to low concentrations. These reproductive disorders can potentially impair the efficiency of applied breeding programs, which may result in considerable economic loss.5 Most interestingly, the effects of ZEA exposure on the reproductive system of females have been well documented, whereas data regarding ZEA-in-
duced effects on the reproductive system of males are comparatively limited, with conclusions drawn from both in vivo and in vitro experiments. Specifically, high concentrations of ZEA are reported to have a detrimental effect on reproductive variables in acutely exposed male rats. Oral intake of ZEA by young boars causes decreases in spermatogenesis, testes weight, libido, and circulating testosterone concentrations, whereas mature boars seem to be nonresponsive to ZEA at concentrations as high as 200 mg/kg of body weight. Similarly, subchronic exposure of adult rams to 12 mg of ZEA/d in feed did not appear to compromise their spermatozoa output and quality. Under in vitro conditions, ZEA has been reported to adversely affect some boar and stallion spermatozoa variables associated with fertilizing capacity.

Rabbit production is considered a sector of animal production that has gradually evolved throughout the last decade. Breeding programs have been intensified; artificial insemination is being systematically implemented and acceptable sperm quality is a prerequisite for its success. Various mycotoxins have been reported to affect reproductive endpoints in rabbits. However, it is noteworthy that a paucity of data exist regarding the impact of ZEA on the reproductive system of this species. Subacute oral exposure to ZEA at doses of 0.1, 1.0, and 2.0 mg/kg of body weight has been found to exert potent estrogenic action on rabbit does and seems to affect fertility-associated factors of the early pre-implantation period. With regard to rabbit bucks, there is no available information about the distribution of estrogen receptors in their reproductive organs. Synergistic and additive responses to ZEA, deoxynivalenol, and fumonisins B1 after combined exposure of rabbit bucks have been reported and involve adverse effects on various reproductive end points. Nonetheless, the individual effect of ZEA on rabbit bucks’ reproductive system has not been investigated in depth, to our knowledge.

The objective of the study reported here was to evaluate the effect of ZEA administered orally at an environmentally relevant concentration (50 µg of ZEA/kg of body weight or 157 X 10^-9 mol of ZEA/kg of body weight) on the reproductive system of subchronically exposed rabbit bucks by assessment of spermatozoa quality variables and serum testosterone concentration as major end points. Taking into account the recommendation of the European Union (2006/576/EC) for the maximum allowable concentration of ZEA in complete feeds of prepubertal gifts (0.1 mg/kg) and the fact that rabbit does are highly susceptible to the estrogenic effects of ZEA at doses of 0.1, 1.0, and 2.0 mg/kg of body weight, a lower ZEA daily dose (50 µg/kg of body weight), which is representative of a level of exposure that can be achieved through consumption of regular animal feed, was selected to investigate ZEA’s subchronic effects on rabbit bucks.

Materials and Methods

The study was approved by the General Assembly of the School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece (permission No. 26/12.03.2013), and the Directorate of Veterinary Services, Region of Central Macedonia, Department of Veterinary Appreciation of Medicine and Applications, Thessaloniki, Greece (permission No. 195297/1265/02.07.2015).

Animals

Eight sexually mature New Zealand White rabbit bucks (Oryctolagus cuniculus) were used in the study. The rabbits were 10 months of age; their mean ± SD body weight was 4.0 ± 0.5 kg, and all were considered healthy on the basis of clinical examination and basic laboratory findings. All rabbits had been trained to service an artificial vagina. Animal handling and husbandry were in accordance with the European Community Standards on the Care and Use of Laboratory Animals (Directive 86/609). The bucks were housed individually in wire mesh cages in a common ventilated facility with a constant light-dark cycle (16 hours of light and 8 hours of darkness), an ambient temperature of 16° to 18°C, and relative humidity of 50%. Feed and water were offered ad libitum throughout the study.

The rabbits were fed a commercially available standard pellet diet. Samples of the latter had been previously screened for ZEA and deoxynivalenol contamination; concentrations of both were less than the high-pressure liquid chromatography assay’s detection limits (< 10 µg/kg of feed and < 50 µg/kg of feed, respectively). A 2-week acclimatization period under the aforementioned standard conditions was allowed to elapse prior to commencement of the experimental procedures in the study rabbits.

Mycotoxin solution

The mycotoxin solution was prepared by suspension of lyophilized powder of 99% purified ZEA in DMSO (1,099 X 10^-6 mol of ZEA/L to 1,413 X 10^-6 mol of ZEA/L of solution). Stock aliquots were stored at -20°C until use. Potential DMSO-induced effects on rabbit bucks’ spermatozoa quality and basal serum testosterone concentration were evaluated in a 5-week pre-experimental study (directly following the acclimatization period), during which 0.5 mL of DMSO was administered orally once daily to each of 6 additional bucks; the effects of DMSO were subsequently considered insignificant (data not shown).

Experimental design

The experimental period (extending from March to June) was equally allocated into a 7-week control period followed by a 7-week treatment period. The durations of the control and treatment periods were approximations of a complete spermatozo-
gonic cycle (48 days) in rabbit bucks. Throughout the control period, bucks received water orally by means of a syringe at a dosage of 0.5 mL/buck/d. Ejaculates were collected at 7-day intervals for the evaluation of spermatozoa kinetics (by CASA), morphology, viability, and DNA fragmentation. For each rabbit, blood sample (4 mL) collection (via jugular venipuncture) for the determination of basal serum testosterone concentration was conducted once weekly at a standard hour in the morning and with a 2-day interval between blood sample collection and semen sample collection. During the treatment period, rabbits were treated orally with a daily dose of 0.5 mL of ZEA-DMSO solution. Physical examination of the bucks was performed on a daily basis; the semen and blood sample collections were scheduled as in the control period.

**Semen sample collection**

Sexual stimulation of each rabbit buck was achieved by the introduction of a teaser doe in the rabbit's cage. Ejaculates were collected by means of a glass artificial vagina. Before collection of the ejaculate, the artificial vagina was heated to approximately 40° to 45°C. For each buck, 2 consecutive mounts of the doe were allowed before ejaculate collection. Ejaculates with gel or cellular components were discarded, and another sample was collected within 30 minutes. Semen samples were promptly diluted with a commercial semen extender (vol:vol, 1:1) and were then transported in an isothermic container (18°C) to the laboratory (Unit of Biotechnology of Reproduction, School of Veterinary Medicine, Aristotle University of Thessaloniki) for evaluation within 1 hour after collection.

**Blood sample handling**

On each occasion, a plastic tube (4 mL) containing no anticoagulant was filled with the blood sample. After the blood was allowed to clot, the sample was centrifuged at 1,300 × g for 7 minutes to obtain serum. Each serum sample was placed in a 1.5-mL Eppendorf tube and kept at −80°C for up to 20 days until analysis.

**Determination of basal serum testosterone concentrations**

Serum testosterone concentration was determined by means of an automated analyzer that used a solid-phase competitive chemiluminescence immunoassay. The assay’s lowest detection limit was 0.01 nmol/L, and its use for assessment of serum testosterone concentration in rabbits had been formerly validated.

**Assessment of spermatozoa motility and kinetics**

Spermatozoa motion characteristics (percentages of progressive and total motility; percentages of rapidly, moderately, and slowly moving spermatozoa; velocity variables [VCL {μm/s}, VSL {μm/s}, VAP {μm/s}, linearity {VSL:VCL ratio}, straightness {VSL:VAP ratio}, and wobble {VAP:VCL ratio}]; ALH {μm}; and BCF [Hz]) were evaluated with by use of an automated CASA system and an optical phase-contrast microscope; the microscope was equipped with a digital camera and a heated stage set at 37°C. Specifically, CASA was configured as follows: ≤ 10 fields and > 500 spermatozoa; 50 frames/s; particle area, 10 to 80 μm²; progressive movement, > 70% of the straightness; circular movement, < 50% of the linearity; and depth of field, 10 μm. Spermatozoa moving < 10 μm/s were considered slowly moving, those moving 11 to 25 μm/s were considered moderately moving, and those moving 26 to 50 μm/s were considered rapidly moving. Ten microliters of each prewarmed (37°C) semen sample was loaded on a prewarmed counting chamber and covered with a glass cover slip, avoiding any air-bubble formation. After 60 seconds (to allow cell settling), a minimum of 500 spermatozoa (frames of at least 4 random fields at 100X) were evaluated for kinematic analysis. Each measurement was replicated for reliability purposes, and their mean value was calculated for evaluation.

**Spermatozoa morphology**

Spermatozoa morphology was evaluated by use of a 2-step staining method, in accordance with the manufacturer’s instructions. A minimum of 200 spermatozoa was assessed at 400X under a light microscope; percentages of morphologically normal spermatozoa and spermatozoa with morphological abnormalities of the head, midpiece, and tail compartment were calculated.

**Spermatozoa viability**

Spermatozoa viability in each semen sample was determined by evaluation of cell membrane integrity status, which was performed with calcein (acetoxymethyl ester) and propidium iodide dual fluorescent stain. Briefly, 5 μL of the calcein (acetoxymethyl ester) solution and 1 μL of the propidium iodide solution were added to 100 μL of a semen sample and incubated in the dark for 10 minutes at 38°C. A total of 200 spermatozoa were assessed at 1,000X under a fluorescence microscope. The percentage of live spermatozoa was calculated as the percentage of spermatozoa that had green fluorescence.

**Spermatozoa DNA integrity**

The integrity of spermatozoa DNA was evaluated by use of the acridine orange test as described by Tsakmakidis et al. Acridine orange fluorescence shifts from green (normal, double-stranded DNA) to yellow, orange, or red when DNA denaturation has occurred. For each semen sample, a total of 200 spermatozoa were examined in ten 1,000X fields under a fluorescence microscope. The percentage of DNA-fragmented spermatozoa was calculated as the...
percentage of spermatozoa that had a fluorescence spectrum from yellow to red.

Euthanasia protocol

All rabbits were euthanized at the end of the study by IV administration of 0.6 mL of ketamine hydrochloride and 0.3 mL of dexmedetomidine, followed by IV administration of 5 mL of propofol and 5 mL of potassium chloride solution. Carcasses were promptly necropsied.

Gross and histologic examination of testes and epididymides

Following euthanasia of each rabbit, both testes and epididymides were removed and promptly evaluated for any macroscopic lesions. Specimens were fixed in Bouin fluid and subsequently embedded in wax paraffin. Microtome sections (4 to 6 µm) were obtained. The sections were stained with H&E stain and examined at 400X under a light microscope. Sections of similar tissues from a euthanized healthy rabbit buck were used as controls.

Statistical analysis

The sample size estimation and the statistical analysis were performed with a commercially available statistical package. Sample size analysis preceded the experimentation and was performed with a t test for paired samples. To conduct this analysis, we specified a difference of 10% in the investigated variables, a significance level of 0.05, and a power of 0.85. The mean values and SDs used in this analysis were estimated on the basis of bibliographical data. The normality of the data was tested with the Shapiro-Wilk test. Variables that did not follow a normal distribution were normalized by square-root transformation. For reasons of clarity, the means and SEM of the nontransformed data are reported. Statistical analysis was conducted with a repeated-measures mixed model. The model included period (control vs treatment), week, and their interaction as fixed effects. Rabbit was defined as the subject of the repeated observations. Covariance structure was chosen on the basis of values of the Akaike information criterion. Five models were run with different structures (compound symmetry, unstructured, first-order autoregressive, first-order antidependence, and Toeplitz), and the model with the least Akaike information criterion was chosen. Pairwise comparisons were adjusted by incorporating the Tukey adjustment. A significant difference was defined as a value of \( P < 0.05 \).

Results

Comparison of the findings for the control and treatment periods revealed no significant \( (P > 0.05) \) differences for the percentages of total motile, progressively motile, or rapidly, moderately, and slowly moving spermatozoa (Table 1). Spermatozoa velocity variables (VCL, VSL, VAP, straightness, linearity, and wobble) and ALH did not differ significantly \( (P > 0.05) \) between the control and treatment periods (Table 2). However, BCF significantly \( (P = 0.05) \) increased during the treatment period.

Although the mean percentages of morphologically abnormal spermatozoa were not significantly \( (P = 0.24) \) different between the control and treatment periods (Table 3), there was a pattern of increasing values throughout the treatment period (Table 1). Indeed, significantly \( (P < 0.001) \) higher percentages of spermatozoa with head and midpiece abnormalities were detected during ZEA exposure, compared with findings during the control period. Conversely, the percentages of live spermatozoa were comparable between the control and treatment periods \( (P = 0.45; \text{Table 4}) \). However, in terms of spermatozoa DNA integrity, a significant \( (P = 0.04) \) increase in the

Table 1—Mean ± SEM spermatozoa motility in semen samples collected from 8 sexually mature New Zealand White rabbit bucks at weekly intervals following once daily oral administration of water (0.5 mL; control period) for 7 weeks and then once-daily oral administration of ZEA in DMSO (50 µg of ZEA/kg of body weight [0.5 mL]; treatment period) for 7 weeks.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Period</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total motility (%)</td>
<td>Control</td>
<td>88.4 ± 2.9</td>
<td>91.7 ± 5.3</td>
<td>92.6 ± 3.4</td>
<td>92.8 ± 2.5</td>
<td>96.6 ± 1.4</td>
<td>96.7 ± 1.3</td>
<td>97.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>92.3 ± 1.8</td>
<td>92.4 ± 3.4</td>
<td>97.8 ± 1.0</td>
<td>97.1 ± 1.1</td>
<td>97.0 ± 1.5</td>
<td>95.5 ± 2.9</td>
<td>95.7 ± 0.8</td>
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<tr>
<td>Progressive motility (%)</td>
<td>Control</td>
<td>26.3 ± 3.0</td>
<td>24.4 ± 0.9</td>
<td>26.8 ± 3.1</td>
<td>26.9 ± 2.6</td>
<td>26.5 ± 1.9</td>
<td>32.7 ± 2.9</td>
<td>30.8 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>26.8 ± 1.0</td>
<td>30.0 ± 2.9</td>
<td>34.2 ± 3.6</td>
<td>28.8 ± 1.9</td>
<td>31.8 ± 3.1</td>
<td>32.4 ± 4.2</td>
<td>29.6 ± 2.4</td>
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<tr>
<td>Rapidly moving spermatozoa (%)</td>
<td>Control</td>
<td>41.8 ± 8.0</td>
<td>52.4 ± 10.6</td>
<td>51.6 ± 2.6</td>
<td>53.5 ± 7.7</td>
<td>59.8 ± 5.4</td>
<td>70.0 ± 6.7</td>
<td>74.0 ± 8.8</td>
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<td>Treatment</td>
<td>24.1 ± 1.2</td>
<td>21.0 ± 1.6</td>
<td>14.5 ± 3.0</td>
<td>18.0 ± 3.5</td>
<td>18.2 ± 3.7</td>
<td>16.7 ± 2.2</td>
<td>22.8 ± 3.0</td>
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<td>Moderately moving spermatozoa (%)</td>
<td>Control</td>
<td>22.1 ± 2.0</td>
<td>23.9 ± 3.1</td>
<td>23.9 ± 1.6</td>
<td>23.0 ± 2.7</td>
<td>23.1 ± 2.3</td>
<td>16.4 ± 2.8</td>
<td>15.3 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>24.1 ± 1.2</td>
<td>21.0 ± 1.6</td>
<td>14.5 ± 3.0</td>
<td>18.0 ± 3.5</td>
<td>18.2 ± 3.7</td>
<td>16.7 ± 2.2</td>
<td>22.8 ± 3.0</td>
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<tr>
<td>Slowly moving spermatozoa (%)</td>
<td>Control</td>
<td>17.8 ± 2.1</td>
<td>14.1 ± 2.1</td>
<td>7.6 ± 2.3</td>
<td>10.7 ± 2.8</td>
<td>11.1 ± 3.9</td>
<td>10.4 ± 3.4</td>
<td>13.8 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>24.5 ± 3.9</td>
<td>15.3 ± 2.9</td>
<td>17.1 ± 3.3</td>
<td>16.4 ± 3.3</td>
<td>13.6 ± 2.7</td>
<td>10.3 ± 2.8</td>
<td>8.7 ± 4.5</td>
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</table>

Rabbits were housed in individual cages. Water and ZEA-DMSO solution were administered orally by use of a syringe. Once weekly, a teaser doe was introduced into each rabbit’s cage; 2 consecutive mounts of the doe were allowed before ejaculate collection. Ejaculates were collected by means of a preheated glass artificial vagina. Ejaculates with gel or cellular components were discarded, and another semen sample was collected within 30 minutes. Semen samples were diluted with semen extender and transported in an isothermic (18°C) container for evaluation within 1 hour after collection. Spermatozoa motion characteristics were evaluated by use of a CASA system and expressed as percentages. Rapidly, moderately, and slowly moving spermatozoa were classified as follows: ≤ 10 µm/s, slowly moving spermatozoa; 11 to 25 µm/s, moderately moving spermatozoa; and 26 to 50 µm/s, rapidly moving spermatozoa. The variables evaluated did not differ significantly between the control and treatment periods.
Table 2—Mean ± SEM spermatozoa motion variables in semen samples collected from the 8 rabbit bucks in Table 1 at weekly intervals following once-daily oral administration of water (control period) for 7 weeks and then once daily oral administration of 50 μg of ZEA/kg of body weight (treatment period) for 7 weeks.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Period</th>
<th>1</th>
<th>2</th>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
<td>VCL (μm/s)</td>
<td>Control</td>
<td>60.6 ± 8.2</td>
<td>63.4 ± 7.2</td>
<td>68.3 ± 6.3</td>
<td>74.0 ± 8.2</td>
<td>74.9 ± 5.5</td>
<td>92.7 ± 8.9</td>
<td>96.5 ± 11.1</td>
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<td>Treatment</td>
<td>66.8 ± 3.9</td>
<td>75.3 ± 6.0</td>
<td>96.7 ± 7.3</td>
<td>89.0 ± 11.7</td>
<td>82.7 ± 8.6</td>
<td>86.7 ± 8.5</td>
<td>78.5 ± 7.3</td>
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<tr>
<td>VSL (μm/s)</td>
<td>Control</td>
<td>21.6 ± 4.1</td>
<td>18.1 ± 2.0</td>
<td>22.4 ± 2.8</td>
<td>23.9 ± 3.7</td>
<td>22.6 ± 3.5</td>
<td>32.1 ± 3.7</td>
<td>33.2 ± 6.1</td>
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<td></td>
<td>Treatment</td>
<td>21.6 ± 1.4</td>
<td>25.7 ± 3.2</td>
<td>33.8 ± 3.6</td>
<td>28.1 ± 4.5</td>
<td>26.8 ± 3.9</td>
<td>30.3 ± 4.5</td>
<td>26.8 ± 3.4</td>
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<tr>
<td>VAP (μm/s)</td>
<td>Control</td>
<td>34.3 ± 5.8</td>
<td>32.7 ± 4.3</td>
<td>37.3 ± 4.1</td>
<td>39.0 ± 5.3</td>
<td>39.0 ± 3.7</td>
<td>52.1 ± 5.5</td>
<td>55.3 ± 8.1</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>35.5 ± 2.4</td>
<td>41.0 ± 4.6</td>
<td>54.3 ± 5.3</td>
<td>48.0 ± 7.6</td>
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<td>Control</td>
<td>34.7 ± 2.6</td>
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<td></td>
<td>Treatment</td>
<td>32.5 ± 1.8</td>
<td>33.4 ± 1.9</td>
<td>34.2 ± 2.2</td>
<td>30.9 ± 1.8</td>
<td>31.2 ± 1.9</td>
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<td>Straightness</td>
<td>Control</td>
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<td>56.3 ± 2.0</td>
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<td>58.4 ± 1.3</td>
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<td>Wobble</td>
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<td>56.1 ± 2.0</td>
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<td>Treatment</td>
<td>53.4 ± 2.5</td>
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<td>55.2 ± 2.1</td>
<td>52.7 ± 2.3</td>
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<td>ALH (μm)</td>
<td>Control</td>
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<td>2.2 ± 0.1</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.3 ± 0.2</td>
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<tr>
<td>BCF (Hz)*</td>
<td>Control</td>
<td>9.1 ± 1.9</td>
<td>6.5 ± 1.4</td>
<td>10.5 ± 1.2</td>
<td>11.6 ± 2.0</td>
<td>8.9 ± 1.1</td>
<td>13.2 ± 2.1</td>
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<tr>
<td></td>
<td>Treatment</td>
<td>9.6 ± 1.5</td>
<td>14.0 ± 1.0</td>
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<td>12.0 ± 1.8</td>
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</tbody>
</table>

Spermatozoa motion variables (VCL, VSL, VAP, linearity [VSL:VCL ratio], straightness [VSL:VAP ratio], wobble [VAP:VCL ratio], ALH [μm], and BCF [Hz]) in collected semen samples were evaluated by use of an automated CASA system and an optical phase-contrast microscope. Ten microliters of each prewarmed (37°C) semen sample was loaded on a prewarmed counting chamber and was covered with a glass coverslip, avoiding any air-bubble formation. After 60 seconds (to allow cell settling), a minimum of 500 spermatozoa (frames of at least 4 random fields at 100X) were evaluated for kinematic analysis. Each measurement was replicated for reliability purposes, and the mean value was considered for evaluation. Mean BCF for the control and treatment periods differed significantly (P = 0.05). The other spermatozoa motion variables evaluated did not differ significantly between the control and treatment periods.

See Table 1 for remainder of key.

Table 3—Mean ± SEM spermatozoa quality variables in semen samples collected from the 8 rabbit bucks in Table 1 at weekly intervals following once-daily oral administration of water (control period) for 7 weeks and then once-daily oral administration of 50 μg of ZEA/kg of body weight (treatment period) for 7 weeks

<table>
<thead>
<tr>
<th>Variable</th>
<th>Period</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormal spermatozoa (%)</td>
<td>Control</td>
<td>18.0 ± 7.1</td>
<td>11.0 ± 1.7</td>
<td>15.6 ± 6.8</td>
<td>17.1 ± 6.8</td>
<td>19.0 ± 6.0</td>
<td>17.4 ± 4.8</td>
<td>18.5 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>30.6 ± 5.3</td>
<td>26.1 ± 4.9</td>
<td>27.0 ± 3.1</td>
<td>21.9 ± 2.8</td>
<td>23.6 ± 5.9</td>
<td>28.1 ± 3.4</td>
<td>31.0 ± 10.8</td>
</tr>
<tr>
<td>Spermatozoa head abnormalities (%)</td>
<td>Control</td>
<td>7.0 ± 1.3</td>
<td>6.0 ± 0.4</td>
<td>5.1 ± 1.0</td>
<td>6.1 ± 1.1</td>
<td>7.3 ± 1.0</td>
<td>7.1 ± 0.9</td>
<td>5.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>16.4 ± 2.4</td>
<td>13.6 ± 2.0</td>
<td>13.9 ± 1.3</td>
<td>10.3 ± 1.2</td>
<td>10.1 ± 2.1</td>
<td>15.1 ± 2.3</td>
<td>11.4 ± 2.5</td>
</tr>
<tr>
<td>Spermatozoa midpiece abnormalities (%)</td>
<td>Control</td>
<td>1.6 ± 2.0</td>
<td>1.2 ± 3.1</td>
<td>1.6 ± 1.6</td>
<td>1.5 ± 2.7</td>
<td>1.4 ± 2.3</td>
<td>2.1 ± 2.8</td>
<td>1.7 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>3.7 ± 1.2</td>
<td>3.6 ± 1.6</td>
<td>7.6 ± 3.0</td>
<td>7.3 ± 3.5</td>
<td>7.3 ± 3.7</td>
<td>4.4 ± 2.2</td>
<td>4.3 ± 3.0</td>
</tr>
<tr>
<td>DNA acid-fragmented spermatozoa (%)</td>
<td>Control</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.6 ± 0.3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>0.4 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>1.0 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>1.1 ± 0.4</td>
<td>0.6 ± 0.3</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

Spermatozoa morphology was evaluated by use of a 2-step staining method. In each semen sample, a minimum of 200 spermatozoa was assessed at 400X under a light microscope; percentages of morphologically normal spermatozoa and spermatozoa with morphological abnormalities of the head and midpiece were calculated. The integrity of spermatozoa nuclear chromatin was evaluated by use of the acridine orange test. For each semen sample, a total of 200 spermatozoa were examined in ten 1,000X fields under a fluorescence microscope. The percentage of DNA-fragmented spermatozoa was calculated as the percentage of spermatozoa that had a fluorescence spectrum from yellow to red.

a,b—For a given variable, values during the control and treatment periods differed significantly as follows: a, P < 0.001; b, P = 0.04.

See Table 1 for remainder of key.

percentage of DNA-fragmented spermatozoa was observed during the ZEA treatment period.

No ZEA-induced changes (P > 0.05) in serum testosterone concentration were identified during the mycotoxin exposure period, compared with findings during the control period (Table 4). Gross examination of the rabbits’ testes and epididymides revealed no alterations in macroscopic appearance. Histologic examination of the aforementioned organs did not reveal any abnormal patterns in the seminiferous and epididymal epithelium, compared with findings for control tissue sections.

Discussion

Published data, which pertain mostly to mice and boars, indicate that exposure to ZEA affects male reproductive end points in a dose-dependent manner during both acute and chronic exposures.6,9 Furthermore, it has been suggested that prolonged exposures to low concentrations of mycotoxins can have a del-
Spermatozoa viability in each semen sample was determined by evaluation of cell membrane integrity status, which was performed with calcein (acetoxymethyl ester) and propidium iodide dual fluorescent stain. For each semen sample, a total of 200 spermatozoa were assessed at 1,000X under a fluorescence microscope. The percentage of live spermatozoa was calculated as the percentage of spermatozoa that had green fluorescence. Spermatozoa tail abnormalities were evaluated by use of a 2-step staining method. In each semen sample, a minimum of 200 spermatozoa were assessed at 400X under a light microscope; the percentage of spermatozoa with morphological abnormalities of the tail component was calculated. A blood sample (4 mL) was collected into a tube containing no anticoagulant, allowed to clot, and subsequently centrifuged to obtain serum. Each serum sample was placed in a 1.5-mL Eppendorf tube and stocked at −80°C for up to 20 days until analysis. Basal serum testosterone concentration was determined by means of an automated analyzer that used a solid-phase competitive chemiluminescence immunoassay (lower limit of detection, 0.01 nmol/L) validated for use in rabbits. The variables evaluated did not differ significantly between the control and treatment periods.

See Table 1 for remainder of key.

terious effect on the reproductive system of various species, among which rabbits are included.18 Considering the aforementioned findings and in view of the limited available data for rabbits, we decided to study the effect of subchronic oral exposure to a low concentration of ZEA (as might naturally occur) on reproductive end points of rabbit bucks.

As inferred from the results of the present study, ZEA appears to affect, albeit mildly, some quality variables of rabbit bucks’ spermatozoa. It is noteworthy that ZEA concentrations as low as that used in the present study are not commonly evaluated in vivo with respect to male reproduction. To our knowledge, ZEA exposure has been previously studied at a concentration as low as 8.5 to 9.7 µg/kg of body weight in rabbit bucks, in a context of combined mycotoxin exposure.18 In that investigation, however, more pronounced effects were noted on the reproductive end points of interest, compared with findings of the present study, which is compatible with the notion that exposure to ZEA along with other mycotoxins in nature results in synergistic or additive responses in terms of reproduction.18

Assessment of spermatozoa motility is an integral part of semen analysis because motility is directly related to fertilizing capacity. In the present study, no effects of ZEA exposure on most spermatozoa motion variables were observed, with the exception of mean BCF values, which increased significantly during the 7-week treatment period. Increased BCF is considered indicative of hyperactivated spermatozoa in rats, although its association with fertilization outcome has yet to be clarified.22,23 Exposure to ZEA has been implicated in the induction of hyperactivated motility of stallion spermatozoa under in vitro conditions, which might lead to depletion of spermatozoa energy for the fertilization process.24 However, in the present study, other hyperactivation-associated variables (wobble, VCL, VSL, ALH, and linearity) were concurrently unaffected; thus, a classic hyperactivation or even a non-specific oscillatory movement pattern was not substantiated. Hence, considering that the remainder of the spermatozoa motion variables were not altered, the detected increase in spermatozoa BCF during the treatment period was not considered biologically relevant and could not be further elucidated.

The observed lack of ZEA’s effect on most spermatozoa motility variables in the present study can be considered in a context of equivocal published data. Specifically, the present results are supported by those reported for subchronically exposed rams and boars, in which ZEA administered at concentrations higher than those used in the present study did not affect spermatozoa motility.11,25 Conversely, ZEA has been documented to compromise spermatozoa total motility in acutely exposed (single oral dose of 40 mg of ZEA/kg) mice and subchronically exposed (9 ppm of ZEA in feed) boars.60 Similarly, in vitro ZEA-exposed stallion spermatozoa had decreased VAP and VSL values, whereas ZEA decreased the percentages of progressively motile spermatozoa in boar semen in vitro.12

Normal spermatozoa morphology has been positively correlated with fertility in various species. In rabbits, increased percentages of morphologically abnormal spermatozoa have been negatively correlated with birth rates.26 In the present study, there was a pattern, albeit not significant, of increasing mean percentage of morphologically abnormal spermatozoa throughout the ZEA treatment period. These results were in accordance with those of other ZEA exposure studies27,28 in mice. The pattern of increasing percentage of morphologically abnormal spermatozoa in the present study was a reflection of significant increases in head and midpiece abnormalities. It has been suggested that percentages of spermatozoa with abnormal heads of < 50% do not seem to affect the fertilizing potential of stallions.29 Conversely, as far as rabbits are concerned, there is no published evidence on the extent of the effects of sper-
spermatozoa head and midpiece abnormalities on reproductive potential, to our knowledge. Thus, the results of the present study remain equivocal in terms of their clinical relevance at this time.

The percentage of live spermatozoa, along with spermatozoa motility and concentration, has been reported to account for 16% of the variability in litter size among rabbits. The results of the present study have suggested that ZEA at the studied concentration did not have an effect on spermatozoa viability. However, although there are no published data for rabbits with which to compare the results of the present study, it is known that for swine (a highly ZEA-susceptible species), subchronic exposure to ZEA at a dose of 1 ppm in feed does not significantly affect the percentage of live spermatozoa in boar semen. Moreover, depending on the mycotoxin’s concentration and duration of exposure, a significant or no effect of ZEA on boar semen viability in vitro has been reported.

The regulatory role of testosterone on spermatogenesis, spermatozoa maturation, and libido manifestation has long been established. Zearalenone is thought to modulate steroidogenesis via its targeted toxic effect on Leydig cells, which some authors suggest is triggered by apoptosis. Interestingly, in the present study, subchronic exposure to ZEA at the studied concentration did not seem to compromise serum testosterone concentration in treated rabbits. This finding was at variance with published data, which indicate that circulating testosterone concentration in mice and boars decreases after prolonged exposure to ZEA. However, in those studies, animals were exposed to comparatively higher ZEA concentrations. Along with the absence of adverse changes in circulating testosterone concentration, histologic examination of the rabbits’ testes following ZEA treatment in the present study revealed morphologically normal Leydig cells, and no other pathological changes suggestive of decreased testosterone production were observed.

The detected increases in the percentages of spermatozoa head and midpiece abnormalities during the ZEA treatment period in the present study could imply a weak effect of the mycotoxin on spermatogenesis in rabbits. In this respect, the normal histologic appearance of the seminiferous and epididymal epithelia, which is suggestive of functional testes and normal spermatogenesis, further endorses the assumption that ZEA ingested by rabbit bucks at a dosage of 50 µg/kg of body weight/d might have a mild and eventually reversible effect on spermatogenesis in this species. These results correlated well with those reported by Young et al., which indicated that ZEA induces pathological changes in boars’ testes and epididymides only at high concentrations.

It is maintained that compromised spermatozoa DNA integrity affects breeding efficiency and embryonic development in pigs. On this basis, it is conceivable that in polytocous species, such as rabbits, insemination with semen that has a high number of DNA-damaged spermatozoa negatively affects prolificacy rates. Results of the present study indicated that subchronic exposure of rabbit bucks to ZEA caused a significant increase in the percentages of DNA-fragmented spermatozoa. The mean percentages of DNA-fragmented spermatozoa during the ZEA treatment period (up to 1.1%) were perhaps too low per se to assume any potential adverse effects on fertility. This issue has not been investigated in rabbits, to our knowledge, but in boars, fertility threshold values for DNA-damaged spermatozoa are 6% to 8%, whereas in bulls, they are considered to be 10% to 20%. Furthermore, in mice, it has been described that oocytes are able to repair spermatozoa when the extent of DNA damage is as much as 8%. Nonetheless, Boe-Hansen et al. reported that percentages of DNA-damaged spermatozoa of 2.1% to 3.0% in stored boar semen used for artificial insemination can affect litter sizes. Regardless, the increase in DNA-damaged spermatozoa noted during ZEA exposure in the rabbits of the present study correlated well with published evidence, although that evidence was derived solely from in vitro experiments on boar and stallion semen.

It is noteworthy that, throughout the experimental period, there were some differences observed between consecutive measurements for the percentages of spermatozoa with tail abnormalities and for the percentages of live spermatozoa. According to relevant reports, serial measurements of morphologically abnormal and live spermatozoa percentages are variable within experimental subjects. Therefore, considering that both the experimental conditions and rabbits were constantly monitored throughout the study, biological variability of those variables might account for the aforementioned differences.

In the present study, ZEA administered orally once daily at a dose of 50 µg/kg of body weight appeared to affect some spermatozoa quality variables in subchronically exposed rabbit bucks and induced a reversible effect on spermatogenesis. Nonetheless, the alterations observed were otherwise considered mild and unlikely to compromise the fertility of rabbit bucks. Taking into account the synergistic or additive action of mycotoxins, it is suggested that exposure to higher ZEA concentrations or to a combination of mycotoxins might have a more pronounced effect on this species’ reproductive potential. The impact of ZEA on the reproductive system of rabbit bucks merits further investigation.

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**Footnotes**

a. Sigma-Aldrich Corp, St Louis, Mo.
b. MRABit, Kubus SA, Madrid, Spain.
c. Eurotubo, Trujillo, Peru.
References


