Effects of zearalenone in prepubertal gilts

Letícia C. Teixeira2*, Fabiano Montiani-Ferreira2, Rosângela Locatelli-Dittrich2, Elizabeth Santin2 and Gerald C. Alberton2*


Prepubertal gilts were fed with a diet containing zearalenone (ZEA) in a concentration of 0.75 mg/kg for 21 days. The effects of this mycotoxin on morphologic aspects of the reproductive tract as well as on complete blood count (CBC), serum biochemistry analysis (SBA) and humoral immune response against sheep red blood cells (SRBC) were evaluated. There was a significant increase (P<0.05) on the reproductive tract weight, vulvar area, height of the epithelial cells of endometrial glands and uterine mucosa. These results showed the ability of this nonsteroidal mycotoxin in mimicking actions of 17β estradiol at the concentration of 0.75mg/kg. No changes in weight gain, CBC, SBA parameters and humoral response against SRBC were observed.

INDEX TERMS: Swine, zearalenone, mycotoxin, uterus, vagina, humoral immune response.

RESUMO.- [Efeitos da zearalenona em leitonas pré-púberes.] Leitonas pré-púberes foram alimentadas com ração contendo 0,75mg/kg de zearalenona (ZEA) durante 21 dias. Os efeitos da micotoxina foram avaliados nos aspectos morfológicos do trato reprodutivo, bem como na hematologia, bioquímica sérica e resposta imune hormonal contra hemácias de carneiro. Foi observado aumento significativo (P<0.05) no peso do trato reprodutivo, na área vulvar, na altura das células epiteliais das glândulas endometrais e superficiais da mucosa uterina. Estes resultados demonstraram a capacidade desta micotoxina não esteróide em imitar as ações do 17β estradiol na concentração de 0,75mg/kg. Entretanto, apesar das evidentes alterações nos parâmetros estudados no trato reprodutivo, não foram observadas alterações no ganho de peso, bem como nas avaliações hematológicas e bioquímicas sanguíneas e na resposta imune hormonal contra hemácias de carneiro.

TERMOS DE INDEXAÇÃO: Suínos, zearalenona, micotoxina, útero, vagina, resposta imune hormonal

INTRODUCTION

Corn is highly susceptible to contamination by Fusarium spp. fungi that can produce many mycotoxins (Kumar et al. 2008). Zearalenone (ZEA) stands out among these by its frequent occurrence in southern Brazil (Salay & Mercadante 2002), where high temperature variations provide necessary conditions for mycotoxin production (Martins & Martins 2002). ZEA is a nonsteroidal mycotoxin with estrogenic effects, which can cause reproductive disorders. Pigs are particularly exposed to ZEA by having their diet usually consisting of corn and by being particularly sensitive to its toxic effects (Malekinejad et al. 2006). Comparing to other species, pigs are especially sensitive to ZEA intoxication because the hepatic biotransformation of this mycotoxin results in a higher production of α-zearalenol in this species, which is even more toxic metabolite than ZEA (Kuiper-Goodman et al. 1987, Fink-Gremmels & Malekinejad 2007). Additionally, the α-zearalenol has a higher affinity to bind to estrogen receptors when compared to ZEA and the other metabolite β-zearalenol (Olsen et al. 1981, Malekinejad et al. 2006).

The clinical scenario of intoxication by ZEA varies according to the amount of toxins ingested, period of ingestion, gender, and reproductive stage of each patient. The signs can often be confused with a number of other diseases or management problems. However, vulvar edema and hyperemia are classic signs of this type of intoxication by ZEA (Mostrom et al. 2007). Most studies of experimental intoxication with ZEA use dosages exceeding 1 mg/kg (Etienne & Jemmali 1982, Speranda et al. 2006, Andretta et al. 2008). Doll et al. 2004)
however, found that pathologic changes started to appear at doses as low as 0.42 mg/kg. Additionally, it is known that the occurrence of clinical signs depends on individual factors, dosage, and its clinical manifestation is conditioned to previous stress suffered by exposed animals (Smith et al. 2005). Under experimental conditions, due to greater control of stress factors, proper management, and low challenge, mainly related to infectious pressure; clinical signs were not evident, especially when using very low concentrations of ZEA. This study aimed to evaluate the effects of ZEA in the diet of prepubertal gilts, in a controlled environment. Thus, for the present study, a ZEA concentration of 0.75mg/kg was chosen, which was thought to be high enough to cause pathologic changes and relatively close to concentrations routinely reported in the field as capable of causing intoxication (Vargas et al. 2001, Doll et al. 2004). The toxic effects produced were evaluated using a comparison of reproductive, hematological and biochemical parameters, weight gain of the animals, and humoral immune response against sheep red blood cells (SRBC).

MATERIALS AND METHODS

Animals. Twelve 32-day-old gilts were used on the experiment. General health status of the animals was verified by clinical, hemotological, and biochemical blood test. All gilts were from Danbred® line, with an average weight of 8.55±0.60 kg on the first day of the experiment. The investigation was conducted in 21 days. The animals had one week of housing and management adaptation before the experimental phase. For the experiment, the population was divided into two groups of six animals that received two distinct treatments: T1 (control diet) and T2 (experimental diet with 0.75mg/kg of ZEA). They were housed in individual stalls of plastic floor (2m length x 1m width).

The ZEA concentrate was added in the diet at 0.75mg/kg. After mixing and preparation of the final diets, samples of corn and soybean meal used to produce the diet as well as final feed samples were sent for mycotoxicological analysis at the Laboratory of Mycotoxins (LABMIC) at the Department of Agribusiness, Food and Nutrition ESALQ/USP being analyzed by thin layer chromatography. The concentration of ZEA and ochratoxin were determined by the modified method of Soares and Rodriguez-Amaya (1989) with immunoaffinity column purification. The concentration of aflatoxins B1, B2, G1, G2 were determined by the MAPA method (Ministry of Agriculture and Livestock) Brazil (2000). The correction of calculation was performed to obtain the final concentration of ZEA at 0.75mg/kg. After mixing and preparation of the final diets, samples of each treatment were also analyzed for the same toxins by the same methods. Food and water were provided to animals ad libitum.

Hematological and biochemical analysis. Blood samples were collected at the first and last day of the experiment. Samples were obtained by puncturing the vena cava with 18G needle adapted to a 20mL syringe. The blood was immediately transferred to tubes with and without EDTA. The blood without EDTA was centrifuged to obtain serum. Serum samples were stored at -20°C until analysis. The enzymes aspartate aminotransferase (AST) and gammaglutamyl transferase (GGT) were determined by kinetic method; urea; and total protein (TP) by biuret method; and albumin by bromocresol green reaction. Analyses were performed on semi-automatic biochemical analyzer CELM-SBA-200.

Table 1. Branatological composition of the basic diet fed to the gilts.

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humidity (%)</td>
<td>10.212</td>
</tr>
<tr>
<td>Crude Protein (%)</td>
<td>20.172</td>
</tr>
<tr>
<td>Crude Fat (%)</td>
<td>4.513</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>6.711</td>
</tr>
<tr>
<td>Crude Fiber (%)</td>
<td>3.279</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.693</td>
</tr>
<tr>
<td>Available phosphorus (%)</td>
<td>0.449</td>
</tr>
<tr>
<td>Lysine (%)</td>
<td>1.287</td>
</tr>
<tr>
<td>ME kcal/kg</td>
<td>3,284.39</td>
</tr>
</tbody>
</table>

ME = Calculated metabolizable energy.

Diet, feeding and weekly management. The diet of the gilts was composed of corn, soybeans, and vitamin-mineral nucleus formulated according to NRC (NRC, 1998), consistent with the phase of the life cycle (Table 1). The corn used for diet preparation was obtained from a supplier who did not perform careful selection of it.

Gilts were monitored twice a day and were weighed every week, obtaining the calculated weight gain. Vulvar measurements (millimeters) were performed weekly with digital calipers. The vulvar area was obtained by multiplying the width (latero-lateral axis, of the vulvar labia majora) and height (dorsal-ventral axis, from dorsal commissure to prepuce of clitoris) measurements of the vulva.

Addition of zearalenone and mycotoxin analysis. The ZEA (Sigma Aldrich Corporation) had 99% minimum purity and was initially mixed in degeminated corn in the process of serial dilution. A mixture of degeminated corn meal + ZEA was incorporated into the diet with a “Y” type mixer. Samples of corn and soybean meal used to produce the diet as well as final feed samples were sent for mycotoxicological analysis at the Laboratory of Mycotoxins (LABMIC) at the Department of Agribusiness, Food and Nutrition ESALQ/USP being analyzed by thin layer chromatography. The concentration of ZEA and ochratoxin were determined by the modified method of Soares and Rodriguez-Amaya (1989) with immunoaffinity column purification. The concentration of aflatoxins B1, B2, G1, G2 were determined by the MAPA method (Ministry of Agriculture and Livestock) Brazil (2000). The correction of calculation was performed to obtain the final concentration of ZEA at 0.75mg/kg. After mixing and preparation of the final diets, samples of each treatment were also analyzed for the same toxins by the same methods. Food and water were provided to animals ad libitum.

Morphologic and histopathologic analysis of organs. The animals were slaughtered after being fed with the experimental diets for 21 days in a commercial abattoir. Liver, mesenteric lymph nodes, and reproductive tract including ovaries, uterus, vagina and vulva were examined macroscopically, evaluating general appearance. Reproductive tract were photographed, dissected and weighed. Then, vulva were removed and ovaries, uterus and vagina were weighted together. The relative weight of the reproductive tract and the ovaries-uterus-vagina complex were calculated, dividing the values of the observed weights by the body weight and multiplying by 100.

Samples of uterus, lymph nodes, and liver were collected and fixed in 10% buffered formalin phosphate, shortly after slaughter. Then samples were processed according to routine histologic techniques, embedded in paraffin and cut to obtain sections of 5 im thickness. The sections were fixed on glass slides and stained with hematoxylin and eosin.

Samples of liver, lymph nodes, uterus and vagina were evaluated for the presence of morphologic changes. Three sections of each uterine horn, collected at mid portion were measured following these criteria: mean height of epithelial cells of the endometrial glands and mean height of epithelial cell of the uterine mucosa surface. These measurements were used as markers for cellular hypertrophy (Heneweir et al. 2007). For this measurement, three
microscopic fields with endometrial glands and three with the epithelial surface layer of the uterine mucosa were photographed with a magnification of 400x, measuring four times in different fields, with the program "Motic Images Plus 2.0®". The vaginal canal histopathologic analysis was performed with fragments from two locations, one from the proximal third of the uterus and other from the distal third, closest to the vulva, due to normal differences in stratification of the epithelium in length, with a reduction of stratification as it approaches the cervix.

**Evaluation of humoral immune response against sheep red blood cell.** The humoral immune response was measured by determining anti-sheep red blood cell plate hemagglutination test following the protocol with two intramuscular inoculations of $10^8$ SRBC diluted in saline and with Freund’s complete adjuvant (modified from Bonnette et al. 1990). The first inoculation was made on the 10th day after the beginning of the experiment and the second inoculation seven days after the first inoculation (17th day of the experiment). Blood samples were collected five days after the first inoculation and four days after the second inoculation. Serum was kept frozen at -20°C in polyethylene tubes for plate hemagglutination test (HA). The hemagglutination titers were determined after inactivation of the serum by serial dilution method according to Schurig et al. (1978).

**Statistical analysis.** The experimental design was completely randomized with two treatments and six replicates. Each animal was considered as an experimental unit. The results of serological analysis for humoral response against SRBC were converted to Log$_{10}$ before statistical analysis. Numeric continuous data between groups were evaluated statistically by one-way ANOVA. When the effect of treatment was detected, a Tukey-Kramer post-hoc test was performed to identify significant differences between means. Differences were deemed significant when P<0.05 (SAS-JMP, version 3.2.2; SAS Institute Inc, Cary, NC).

**RESULTS**

**Mycotoxicological analysis**

Mycotoxicological results are shown in Table 2. ZEA was previously present in milled corn, thus a correction was made to obtain a final concentration at T2 of 0.75mg/kg of ZEA in the diet. A concentration of 0.335mg/kg of ZEA was found in the control group. Traces of aflatoxin B1 (0.014mg/kg) were detected in the milled corn, previously to final diet formulation. However, it was not detected after preparation of the diet.

**Vulva measurement and reproductive tract weight**

Significant differences of the vulva measurement were observed only in the 21st day of experiment (Fig.1). The weights of the reproductive tracts and ovaries-uterus-vagina complexes of the group with ZEA (T2) were significantly higher than the control group (T1) (Fig.2).

**Epithelial cells height of the endometrial glands and uterine mucosa surface**

Results of height measurements of epithelial cells of endometrial glands and uterine mucosa surface are shown in
Fig. 4. Uterus of gilts. HE, 400x, T1 (A and C, control) and T2 (B and D, 0.75mg/kg ZEA). (A) Regular epithelial layer of uterine mucosa. (C) Uniform and regular epithelial cells of endometrial glands. (B) Irregularity, thickening and proliferation of the uterine epithelial cells (arrow), squamous metaplasia of the uterus. (D) Hyperplasia of the endometrial glands (proliferation of epithelial cells with irregular increase of epithelial layers) (arrow).

Fig. 5. Vaginal canal of gilts, HE, T1 (A and B, control) and T2 (C and D, 0.75mg/kg ZEA). Epithelium near cervix (A and C). Epithelium near vulva (B and D). (A) Uniform and regular epithelial cells (x200). (B) Regular stratification (x100). (C) Epithelium with irregular cellular proliferation (arrow) (x100). (D) Irregular stratified epithelium (arrow) (x100).
Morphological and histopathologic analysis of the organs

No macroscopic changes were observed in the examined organs, except in two animals of T2 which had cystic ovaries.

On histopathologic evaluation, from both treatments, the mesenteric lymph nodes were within normal characteristics. There was mild biliary hyperplasia in samples of liver in T1 and T2. The histologic analysis of the reproductive tract showed proliferation (hyperplasia) of the epithelial cell layer of the uterine (Fig.4) and vaginal mucosa (Fig 5), resulting in thickening and irregularity of the epithelium from gilts of T2 group. Squamous metaplasia in uterus and hyperplasia of the endometrial glands (Fig.4) were also observed.

Weight gain

No differences were observed between the mean total weight gain (11.96 and 12.59 kg) of T1 and T2 groups (P>0.05).

Hematological and biochemical analysis and humoral immune response against SRBC

There were no significant differences between treatments for hematologic and serum biochemistry analysis and in the titers for immunization with SRBC.

DISCUSSION

Mycotoxicological analysis results suggest frequent occurrence of ZEA in Brazilian grains (Salay & Mercadante 2002), since this mycotoxin was found in the regular corn and soybean meal used in this experiment.

Since this mycotoxin was found in the regular corn and soybean (T1), the hematological and biochemical analyses were performed to evaluate the effects of ZEA on animal performance. The hematological and biochemical analyses showed no significant differences between groups.

The presence of mild hyperplasia of bile ducts in both treatments may be related to the fact that in T1 ZEA concentration was of 0.335mg/kg. ZEA metabolism in the liver may also be indicative of response to mild toxicity. In addition, the presence of aflatoxin in milled corn, even if in low concentration, or the presence of other mycotoxins and toxic agents in the diet that were not surveyed, may cause higher metabolic activity in this organ, including in the group that did not receive additional ZEA, just what was already present in the corn and soybean (T1).

There were no significant differences in blood hematolo-
gical and biochemical parameters between treatments. In gilts exposed to 3mg/kg of ZEA the levels of increase of AST, GGT and lactate dehydrogenase were increased (Speranda et al. 2006). The influence of this intoxication on these parameters are poorly described in the literature for pigs; however, there are studies demonstrating hematotoxic and hepatotoxic actions of ZEA in rats and mice (Maaroufi et al. 1996, Abbès et al. 2006, Stadnik et al. 2009).

The evaluation of SRBC antibody titers was used extensively in the literature (Peplowksi et al. 1980, Bonnette et al. 1990, Rotter et al. 1994) to study the immune response of animals. It is known; however, that the immune response to different challenges is extremely complex. In the challenge with sheep erythrocytes, Rotter et al. (1994) also found no significant difference, but a numerically delayed response of antibody titers of piglets with 5-6 weeks of age fed with low concentrations of DON (0, 0.75, 1.5, 3mg/kg) and ZEA (0, 0.05, 0.08, 0.15mg/kg) was observed.

No histologic changes was observed in lymph nodes, there was no lymphoid depletion, however, mycotoxins can affect immune responses of animals in different ways, from changes in the production of enzymes and cytokines to an increased production of free radicals that indirectly affect these parameters (Surai & Dvorska et al. 2005). Other studies report inhibitory effects of ZEA on the proliferation of lymphocytes, demonstrating its immunotoxic potential at high concentrations. Abbès et al. (2006) observed significant reduction in the number of lymphocytes T cells CD3+, CD4+, CD8+ and CD56+ in mice with 40mg/kg of ZEA. Luongo et al. (2008), in an in vitro study, with low concentrations (2.5μm) of a-zearalenol observed inhibitory effect on cell proliferation of pig lymphocytes. It is believed that this methodology may not have been able to determine the real effect of ZEA on immune response.

The concentration of 0.75 mg/kg of ZEA was sufficient to produce the effect of increasing reproductive tract weight, vulvar area, and causing hypertrophia and hyperplasia of the epithelial cells of the endometrial glands and superficial cells of uterine mucosa as well as vaginal mucosa. Furthermore, no changes on animal body weight gain, hematologic parameters, serum biochemistry analysis and finally, humoral immune response against sheep red blood cells were observed in this investigation.

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REFERENCES


