Effect of zearalenone on the seminiferous tubules structure after dietary exposure in rats

Received for publication, October 20, 2016
Accepted, January 01, 2017

ALEXANDRU RAUL POP1, VIOREL MICLĂUŞ2, ROMEO MICU3*, ALEXANDRU ŞONEA4, ALEXANDRA IRIMIE5, ADRIAN MACRI6, VASILE RUS2, IOAN ŞTEFAN GROZA1
1Clinical Reproduction Department, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania
2Department of Cell Biology, Histology and, Embriology, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania
3Department of Human Assisted Reproduction, 1st Gynecology Clinic, University of Medicine and Pharmacology „Iuliu Hatieganu” Cluj-Napoca, Romania
4Reproduction Department, Faculty of Animal Science and Biotechnologies, University of Agricultural Sciences and Veterinary Medicine Bucharest, Romania
5Pathology Department, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania
6Animal Production and Food Safety Department, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania
*Address for correspondence to: romeomicu@hotmail.com

Abstract

The aim of the current study was to assess zearalenone effect on the seminiferous tubules structure following dietary exposure in Wistar rats. Healthy animals were fed either with diet containing 30g of F. moniliforme and F. graminearum contaminated-maize (46.5µg zearalenone/animal; Zearalenone group, N=21) or with mycotoxin-free diet (Control, N=21), for a period of 60 days. Zearalenone concentration was determined by ELISA. Following orchiectomy, tissue samples were histologically processed, stained by Goldner's Trichrome. In the Control group, normal appearance of the seminiferous tubules and cells in different spermatogenesis stages were observed. In the Zearalenone group, approximately 50% of the seminiferous tubules had cytoarchitectural pathologic changes: projections of the seminiferous epithelium (fold-like papilla shape), tendency of lumen obliteration and intussusception in their own cavity. No spermatozoa were identified in the affected tubules. Intercellular edema, swelling of spermatozoa heads, spermatocytes and spermatids apoptosis, and vacuolar degeneration of spermatocytes were observed. Daily exposure of rats to zearalenone for 60 days affected most of the seminal cell line, selectively induced Sertoli cell swelling/vacuolar degeneration, and occurrence of fold-like papillary structures and intussusceptions of the seminiferous tubules. These findings highlight the zearalenone gametotoxic effect, suggesting the potential risk for the public health following contaminated food consumption.

Keywords: Fusarium species, mycotoxin, contaminated maize, male rats, histopathology.

1. Introduction

Zearalenone is a mycotoxin produced by different Fusarium (F.) species [e.g. F. roseum, F. tricinctum, F. graminearum, F. sporotrichioides, F. oxysporum and F. moniliforme] and has the properties of a non-steroidal estrogenic compound (A. ZINEDINE & al. [1]). Besides its estrogenic effects, it may also have anabolic activity. It has been shown that exposure of domestic animals to zearalenone may cause different reproductive disorders including clinical signs of hyperestrogenism in both females and males subjects. Zearalenone
Toxicoses were often reported in pigs and other livestock animals such as cows, horses and sheep due to widespread occurrence of the mycotoxin in maize, maize-derived products and other cereals. The potential risk of human infection by contaminated cereals and by products of animal origin (e.g. meat, milk, eggs) is intensively discussed (D. Anderson & al. [2]).

Even if zearalenone toxicity may cause different types of reproductive disorders in animals and humans, limited data is currently available regarding zearalenone effect on the seminiferous epithelium.

In order to identify the potentially toxic effect of different agents on the gametogenic function in males, the internationally accepted protocols recommend that animals included in experimental studies to undergo exposure to the toxic agent for a time period at least equal to six times the length of a seminiferous epithelium cycle: 53 days in mice, 52 days in rats, 77 days in hamsters, 64 days in rabbits, 81 days in dogs, 57 days in males from Rhesus family and 96 days in man, for a group of germinal cells to progress from the stage of A1 spermatagonia to spermatozoid (G. Brunborg & al. [3]).

It has been shown that the most sensitive cells to the action of toxic agents are those going through mitosis stage or during DNA synthesis. In case of spermatogenesis may be affected the proliferative spermatogonies or primary preleptotene spermatogonias (B. Robaire & F.B. Hales [4]). There are also several exceptions for example when the toxic agent affects the synthesis of ARN inhibitors in the germinal cells during an advanced stage of differentiation. The most resistant cells to toxic agents are considered type B1 spermatagonia, also known as reserve spermatogonias (L.Y.Y. Sanin & al. [5]).

The aim of the current study was to assess the zearalenone effect on the cytoarchitectural structure of the seminiferous tubules and seminal cell line following dietary exposure in Wistar rats.

2. Materials and Methods

Animals and study design. A total number of 42 Wistar rats were included in this study. They were bred at the Faculty of Veterinary Medicine Cluj-Napoca in accordance with the animal’s standards of care, and the study was approved by the Institute’s Ethics Committee. The animals were housed under standard environmental conditions: around 22°C and 50–70% humidity.

The following criteria were used for selecting the animals included in this study: age, between 5 and 6 months; gender, male; weight, between 250 and 300 g; and physiologic state, healthy and sexually mature (based on clinical examination). The rats were randomly divided into two groups, and were fed for 60 days either with diet containing 30 g of F. moniliforme and F. graminearum contaminated maize, at a concentration of 46.5 μg of zearalenone/animal (Zearalenone group, N=21), either with mycotoxin-free diet based on standard laboratory food for rats (hard pellets from the Cantacuzino Institute, Bucharest, Romania) (Control group, N=21). Water was administrated ad-libitum.

Analytical methods for evaluation the concentration of zearalenone. Zearalenone concentration in contaminated maize was determined by using the enzyme linked immunosorbet assay (ELISA) commercially available kit (RIDASCREEN®FAST Zearalenon, R-Biopharm AG, Bergstraße 17, Darmstadt, Germany), according to the manufacturer’s instructions. Samples were collected using spears and scoops from different layers of the contaminated maize used in this study. Multiple subsamples were pooled, mixed and quartered. The opposite quarters were excluded from the analysis, the remaining sample was mixed, and then a final amount of 500g was used for analysis. These samples were cut in
particles of 1.0 mm. A representative sample was processed for further analysis. Of these, 5 g of ground sample was mixed with 25 ml of methanol (70%). Following 3 minutes of manually shaking, the sample was filtered using Whatman no. 1 filter. Each 1 ml of the obtained filtrate was diluted with 1 ml of distilled water. 50 µl of filtrate were then added in each well of the test. The microplate wells are coated with capture antibodies directed against anti – mycotoxin antibodies. In each well, for both the standard and sample, 50 µl of standard prepared sample was pipetted, and then 50 µl of enzyme conjugate and 50 µl of anti – zearalenone antibody solution were are added. The free mycotoxins and enzyme conjugate compete for the binding sites of coverage antibodies of wells (competitive enzyme immunoassay). The unbound enzyme conjugate were removed during three washing steps with distilled water (250 µl per well). The substrate/chromogen (100 µl) was added to each well and mixed by manually shaking the plate. Following 5 minutes (+/- 0.5 minutes) incubation at room temperature (20–25°C; 68–77°F) in the dark, 100 µl of stop solution (yellow cap) was added to each well revealing after 10 minutes the color transfer from red to blue. The tests’ readings were performed at 450 nm. The absorbance is inversely proportional to the concentration of mycotoxin in the sample.

**Tissue collection and histology.** At the end of the diet period, all rats underwent bilateral orchietomy under general anesthesia. The scrotal approach was used and all animals survived. Testicular tissue was harvested from each animal.

The testis samples were fixed in 10% neutral buffered formalin for 72 hours. They were afterwards rinsed, dried and dehydrated by using gradually increasing concentrations of ethanol (70%, 95% and absolute) successively for 1 day. The tissue samples were cleared with butyl alcohol (n-butanol) and then included in paraffin at 57°C. Serial sections of 5 µ thickness) were performed. They were stained using Goldner's Trichrome method and examined using an optical microscope (20X, 40X).

**3. Results and discussion**

Our study was designed to assess zearalenone impact on the morphological structure and function of seminiferous tubules in rats. Previous studies showed that duration of the seminiferous epithelium cycle in rats is about 8.6 days, and of the spermatogenetic cycle (the time necessary for a germ cell group to develop from the differentiated spermatogonia stage A1 to mature spermatozoa) is about 4.5 times longer (approximately 39 days). In this species, the exposure to potentially toxic agent should be equivalent to six times the length of a seminiferous epithelium cycle (approximately 52 days) (A. ZINEDINE & al. [1]; B. ROBAIRE & F.B. HALES [4]). Here, we report results of zearalenone effect on the gametogenesis following a period of exposure of 60 days. The mean daily dose administrated in rats from the Zearalenone group was 46.5 mg zearalenone/animal. We choose this dose based on previous publications reporting that that this amount was commonly found in the nutritional substrates infested with *F. moniliforme* and *F. graminearum* species (Y.H. JEE & al. [6], K.P. HOYES & al. [7]); this dose is also higher than the minimum dose accepted for a measurable gametotoxic induced-effect in animals. We assessed zearalenone impact by using histopathological examination, a method that is considered to be specific and sensitive enough to detect gametotoxic effect (T. NAGAO [8]; A. EL MAFKAWY & al. [9]).

In the Control group, the histological examination showed that the structure of seminiferous epithelium had normal appearance. Depending on the stage of spermatogenesis, different cell types were observed: spermatogonia, primary and secondary spermatocytes, and spermatids in the seminiferous epithelium, and mature spermatozoa in the lumen of several seminiferous tubules (Figure 1).
In the Zearalenone group, approximately 50% of the seminiferous tubules had unaffected seminiferous epithelium with normal appearance in terms of spermatogenesis and mature spermatozoa, and 50% of seminiferous tubules had different types of pathologic lesions. There were identified cytoarchitectural pathologic changes of the seminiferous epithelium, especially at the level of the epithelium surrounding the lumen. Projections of the seminiferous epithelium with very tall fold-like papilla shape arriving up to the center of seminiferous tubules were identified (Figure 2).

A closely examination of these fold-like tissue structures with proliferating characteristics revealed the presence of cells similar to those found in the basement membrane, near the basal compartment of the seminiferous tubules (e.g. spermatogonias and spermatocytes). An obvious tendency of seminiferous tubules’ lumen obliteration was also observed (Figure 3).

At seminiferous tubules level, more advanced cytoarchitectural pathologic changes were identified, including appearance of tubular formations arranged eccentrically in the seminiferous tubules, but without coming into contact with the basal membrane of the tube, and with its own basal membrane (Figure 4).

These morphological characteristics suggest occurrence of the intussusception phenomenon of the seminiferous tubules in their own cavity.

No spermatozoa were identified in the seminiferous tubules with cytoarchitectural pathologic changes. Although it is possible that some stages of spermatogenesis take place here, it does not seem that the process arrives up to the spermatisds stage. In addition, was also observed intercellular edema with cell separation and accumulation of debris in the lumen of the seminiferous tubules, swelling of sperm heads, spermatocytes and spermatids apoptosis, and vacuolar degeneration of spermatocytes.
These results suggest that zearalenone toxic action induced pathologic changes of the majority of seminal cell line following 60 days administration of the *F. moniliforme* and *F. graminearum*-contaminated maize in sexually mature rats. Contrary to our findings, previous studies conducted in rams and male rats showed that zearalenone administration had no effect on spermatogenesis (G.D. MILANO et al. [10]; G.D. MILANO et al. [11]). Also they have described alteration of the seminal plasma glucose concentration and this may affect the sperm cells motility (Z.TRUTA et al. [12]). It is important to note that compared to these, in our study the period of exposure to zearalenone was approximately 3 times longer and the administered dose was more than double. However, type A1 and B1 spermatogonias (also known as the "reserve" or "resistant" spermatogonias), that are located in the basal compartment of the seminiferous tubules, were less affected.

The degree of pathologic changes was different in the basal compartment of the seminiferous tubules compared with those found in the adluminal area. In the basal compartment a moderate number of spermatogonias and spermatocytes were affected, with considerable differences from one seminiferous tube to another. Due to the dynamic pathologic process, it was not possible to evaluate the exact number of basal cells eliminated following cell apoptosis. In the adluminal area were observed severe changes including cell swelling, vacuolar degeneration, apoptotic bodies and cells in apoptosis; spermiogenesis process was stopped in several areas (Figure 5).

In addition, the proliferative fold-like tissue structures, originating from the basal compartment of seminiferous tubules and arriving up to the adluminal compartment, may be the result of Sertoli syncytials proliferations. It is known that the seminal line cell apoptosis is closely related to Sertoli cell activity (L.Y.Y. SANIN & al. [5]). Vacuolar degeneration in or between Sertoli cells is an early sign of Sertoli cell damage. The vacuoles can be solitary and located in the seminiferous epithelium cell at different levels. Light microscopy is a feasible method to detect whether vacuoles have intra or extracellular location. In some cases, intracellular micro-vacuolization or swelling may affect the basal area of Sertoli cell cytoplasm causing the migration and disorganization of the seminal cells. These changes are suggestive for Sertoli cells and can represent the smooth endoplasmatic reticulum’s changes. Sertoli syncytials may also play a role in the synthesis of basal tonic levels of estrogen in...
testis as they have specific estrogen receptors. However, some studies conducted in rats and boars showed that their role is not significant (G.D. MILANO et al. [10]; A. MANKEVICIENE [13]). Similar results were obtained by JEE et al (2010), who showed that estrogen receptors are not significantly involved in the proliferation of Sertoli cells, suggesting the minim role that they play in the initiation and development of cell mobilization and apoptosis. However, they observed an increase of Fas-Fas ligand values in seminal line cells (haploid) following zearalenone administration, suggesting the involvement of Fas and Fas ligand system in the initiation and evolution of the proliferation processes, cell detachment and apoptosis, mainly affecting the basal cell compartment, spermatogonias and spermatocytes (Y.H. JEE & al. [6]). Fas ligand is a trans-membrane protein with a homotrimeric structure that is part of the tumor necrosis factor family with an affinity for the specific Fas receptor and leads to apoptosis initiation (B. ROBAIRE & F.B. HALES [4], (L.Y.Y. SANIN & al. [5]).

![Figure 5](image1.png)

**Figure 5.** Pathologic changes in the adluminal compartment of the seminiferous tubules in the Zearalenone group
The black arrows indicate spermatocytes in apoptosis and apoptotic bodies of the seminiferous tube (black arrows) (Goldner’s Trichrome, 20X)

![Figure 6](image2.png)

**Figure 6.** Depletion and mobilization of the seminal cell line in the Zearalenone group
The black arrows indicate mechanical obliteration (Goldner’s Trichrome, 20X)

We have also identified intussusception of the seminiferous tubules in their own lumen. This pathologic change may be caused by massive and sudden depletion of sperm cell line, which may lead to partial or total mechanical obliteration of the lumen of affected seminiferous tubules (Figure 6).

The fold-like tissue and intussusception of some seminiferous tubules do not seem to be the results of mycotoxin’s direct action, but rather a secondary phenomenon. In these seminiferous tubules, only the basal compartment remains following destruction and massive mobilization of the seminal cell line. Their walls may become thin and flexible facilitating the folding and intussusception phenomena. These cytoarchitectural abnormalities can cause from narrowing to complete cancellation of the seminiferous tubules, with spermatogenic process perturbation. In several significantly damaged seminiferous tubules, the seminal line cell was completely absent.

Our findings suggest the zearalenone ability to pass both the testicular hematologic barrier (by damaging the basal compartment) and the Sertoli cell barrier (by damaging the adluminal compartment), and its negative effect on spermatogenesis in affected seminiferous tubules.
4. Conclusion

Daily exposure of rats to 46.5 µg of zearalenone for a period of 60 days induced cyto-architectural changes such as occurrence of fold-like papillary structures and intussusceptions of the seminiferous tubules, and affected most of the seminal cell line, inducing moderate apoptosis of spermatogonias and spermatocytes; Sertoli cell swelling and vacuolar degeneration, and moderate cell depletion in the adluminal compartment of seminiferous tubules were also observed.

We consider that these cytoarchitectural abnormalities are irreversible, and affect seminiferous tubules function. However, given that only around 50% of the seminiferous tubules are affected, the organ function is partially kept. Exposure for a longer time period may severely affect the reproductive function. This experimental animal model highlights the gametotoxic effect of zearalenone, suggesting the potential risk for the public health in case of consumption of F. moniliforme and F. graminearum contaminated products.

5. Acknowledgements

This study was supported by CNCSIS-UEFISCSU, project PN II RU-PD code 258, contract no. 181/2010.

References