Melatonin Protects the Histologic Structure of the Uterus in Conditions of Zearalenone Intake

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ABSTRACT

Background: Zearalenone is a widely spread mycotoxin, contaminant of most cereal grains. It has uterotropic, estrogenic and anabolic activity in farm animals. The results are hormonal disbalances as hyperestrogenism, Zearalenone inhibits follicle-stimulating hormone production, thus suppressing ovarian follicle development and ovulation. Also, it induces oxidative stress. Melatonin acts as a potent natural antioxidant and regulates the reproductive function by modification of steroidogenesis. Objective: The present study was conducted to provide detailed qualitative histological analysis of uterus of female rats treated with zearalenone and melatonin and contribute to better understanding of the topic.

Methods: Forty adult, female Wistar rats were equally divided into five groups: Z group – zearalenone, 0.3 mg/kg, i.g.; M group – melatonin, 10 mg/kg, i.p.; ZM group – concomitant application of zearalenone and melatonin in the same dosing regimen, VZ group – zearalenone vehiculum/sunflower oil, i.g. and MZ group – melatonin vehiculum/5% ethanol in Ringer, i.p. Animals were treated daily for 28 consecutive days. After that period, all animals were sacrificed to obtain samples for qualitative histological analysis using the light microscope. Results: Zearalenone led to the alterations of the uterine structures, predominantly in the endometrium that were characterized by metaplasia and hypertrophy of the epithelial cells and hypercellularity of the stroma. In the myometrium, zearalenone induced hypertrophy and hyperplasia of the myocytes. Conclusion: Melatonin, when applied together with zearalenone, blocked the adverse effects of the zearalenone.

Keywords: zearalenone, melatonin, rat, uterus, histology

1. BACKGROUND

Zearalenone or F-2 toxin is a macrocyclic lactone mycotoxin that was first isolated in 1962 as a product of Gibberella zeae fungus species (1). It is a metabolite of Fusarium fungi species, namely F. culmorum, F. equiseti, F. graminearum and F. moniliforme (2). Zearalenone is a widely spread contaminant of cereal grains such as corn, wheat, oats, barley, rice, rye, soya, hops and sesame or other crops like broccoli and cabbage. Contamination starts in the field but fungus growth along with the toxin production and further contamination continue, in particular during storage (3).

Zearalenone has uterotropic, estrogenic and anabolic activity in farm animals. This mycotoxin causes morphological and functional alterations of reproductive organs and damages germinal cells (4). Zearalenone and its derivatives have binding affinity to estrogen receptors in the germinal cell cytoplasm thus mimicking physiological response to the natural estrogen, 17-β-estradiol. In the same time, zearalenone competitively inhibits estrogen binding to the uterine receptors (4, 5). The results are hormonal disbalances as hyperestrogenism, vaginal and rectal prolapse, spontaneous abortion, fetal resorption and infertility in animals. Also, it has been implicated that the exposure to the high doses can cause the precocious puberty (6, 7). Clinical signs of zearalenone toxicosis vary according to the species, age and reproductive status of animal (4, 8).

Apart from rather mild estrogenic activity, zearalenone inhibits follicle-stimulating hormone production, suppresses ovarian follicle...
development and ovulation, while causing pseudo gestation and anestrus through corpus luteum retention and luteotropic effects (9, 10).

Also, zearalenone induces oxidative stress which represents another mechanism of toxicity of this mycotoxin (11).

Melatonin is a lipophilic hormone, isolated and identified by Lerner et al. in 1958. In mammals, it is mainly produced at night by the pineal gland, but there are other, extrapineal sources of this hormone such as retina, lymphocytes, thrombocytes, digestive tract, bone marrow, ovary, testes, lens and skin (12).

Melatonin acts as a potent natural antioxidant, free-radical scavenger and inhibitor of pro-oxidative enzymes (13, 14). Also, it influences the reproductive functions by regulation of the hypothalamus-pituitary gland axis and interference with estrogen synthesis and receptors (14, 15). Thus, melatonin regulates the reproductive function via both, oxidative stress reduction and modification of steroidogenesis (15, 16).

According to the literature review, there are few studies that have focused the attention on possible protective effects of melatonin on zearalenone-induced alterations of reproductive organs. Thus, the present study was conducted to provide detailed qualitative histological analysis of uterus of female rats treated with zearalenone and melatonin and contribute to better understanding of the topic.

2. OBJECTIVE
The present study was conducted to provide detailed qualitative histological analysis of uterus of female rats treated with zearalenone and melatonin and contribute to better understanding of the topic.

3. MATERIAL AND METHODS

Chemicals
In the present study, pure crystalline zearalenone (Sigma-Z-2125 EECNo241-864-O) was used, dissolved in sunflower oil (Oleum helianti) immediately before the application. Melatonin (Sigma-Aldrich, St. Louis, MO, USA) treatment solution (2.5mg/mL) was prepared by dissolving in vehiculum composed of 5% ethanol in Ringer’s solution.

Animals and Experimental Design
The experimental procedures were performed in accordance with the recommendations of the Ethical Committee of the Faculty of Medicine, University of Sarajevo. Forty adult, female Wistar rats were raised and kept under standardized laboratory conditions with 12-h light/12-h dark cycle. Animals were randomized into five groups (eight animals per group). Groups and dosing scheme are shown in the Table 1. Body mass was measured daily for each rat to enable necessary dose adjustments. All the values were recorded into previously prepared evidence lists. The application was performed once daily, at the same time (18-19 h), for 28 consecutive days.

Histological Analysis
At the end of the experimental procedure, all animals were sacrificed by using high concentrations of ether, to obtain uterine tissue samples. After excision, samples were fixed in 10% buffered formalin and embedded in paraffin blocks according to the standard procedure. Five micrometers thick serial sections were cut and stained with hematoxylin and eosin (HE), Azan trichrome stain and periodic acid-Schiff stain (PAS). Qualitative histological analysis was performed using the light microscope with digital camera (Eclipse E400, Nikon), and 40x, 100x and 400x magnification. All three uterine layers/tunics – endometrium, myometrium and perimetrium were analyzed, focusing especially on the endometrial histoarchitecture – epithelium, lamina propria, glands and blood vessels.

4. RESULTS

Group M
Slides showed preserved general organisation of the uterine wall. In the endometrium, surface epithelium was composed of two main cell populations – low columnar ciliated cells and bigger, secretory cells (Figure 1A) along with sporadically seen individual small lymphocytes (Figure 1B). Surface epithelium was continuous with glandular epithelium that made up slightly coiled uterine glands whose bases were reaching basal part of lamina propria. Lamina propria was observed as a relatively narrow zone of highly vascularized loose connective tissue, moderately cellular with lymphocytes, discrete bundles of collagen and elastic fibers and abundant amorphous intercellular substance. Only few stromal cells lost their typical fibroblast form and were found as enlarged, containing abundant cytoplasm, notably large nucleus and prominent eccentrical nucleolus. Myometrium of the uterus in M group was mainly uniform, showing interwoven, helically arranged spindle-shaped smooth muscle cells. Because of the nuclear distances and prominent network of blood vessels, proliferative activity was suspected, while the finding of abundant, vacuolated cytoplasm and large nucleus with fine granular chromatin was suggesting hypertrophy of smooth muscle cells. Uterine surface was covered by serosa, connected to the myometrium via narrow subserosal loose connective tissue (Figure 1C).

Group Z
Qualitative histological analysis of the uterus of female rats treated with zearalenone showed pronounced alterations of the endometrium. Increased cellularity, zones of me-

| Group | Number | Substance | Dose | Mode of administration |
|-------|--------|-----------|------|------------------------|
| VM    | 8      | 5% ethanol in Ringer’s solution | / | i.p. |
| M     | 8      | Melatonin; 5% ethanol in Ringer’s solution | 10mg/kg | i.p. |
| VZ    | 8      | Sunflower oil | / | i.g. |
| Z     | 8      | Zearalenone; sunflower oil | 0.3mg/kg | i.g. |
| ZM    | 8      | Zearalenone; sunflower oil + Melatonin; 5% ethanol in Ringer’s solution | 0.5mg/kg + 10mg/kg | i.g. + i.p. successive administration |

Table 1. Animal grouping and dosing regimen. i.p. – intraperitoneal administration; i.g. – intragastric administration.
taplasia, hypertrophy and pseudostratification were noted within the surface epithelium (Figure 2A). Pseudostratified arrangement was found in the area of hypertrophy, because of the irregular alignment of the epithelial cells’ nuclei. In between these altered areas, epithelium was preserved and composed of columnar cells with euchromatic nucleus and pale, vacuolized cytoplasm. Ciliated cells were found narrower than secretory cells. Their nuclei appeared oval, intensely stained with heterochromatin adjacent to the nuclear envelope. Secretory cells were bigger with spherical, euchromatic nuclei and prominent nucleolus. Stroma appeared hypercellular, especially in the upper portion, with occasional invasion to the surface epithelium. Fibroblasts represented its dominant cell population (Figure 2B). Hypertrophy and hyperplasia with prominent intercellular matrix and perivascular leukocytes infiltration were found in myometrium as well as prominent blood vessels. In the area of hyperplasia, smooth muscle cells appeared smaller and their nuclei were elongated and more closely arranged (Figure 2C). On the surface of the uterus, relatively preserved serosa was found.

**Group ZM**

In the group of animals that were treated with zearalenone and melatonin, typical stratification of the uterine wall was present. Starting from the luminal surface of the organ, endometrium, myometrium and perimetrium were detected (Figure 3A). Simple epithelium that lines the lumen of the uterus was relatively preserved. Epithelial cells appeared from low to tall columnar and extend from the basement membrane to the lumen. The most numerous cells were ciliated that appeared narrower than the secretory cells. Their nuclei were elongated and dark, with heterochromatin adjacent to the nuclear envelope.

Secretory cells were more massive compared to the ciliated cells, with spherical, euchromatic nucleus with prominent nucleolus. Homogeneity of their cytoplasm appeared inconsistent with pale areas that correspond to the secretory granules.

Small areas of epithelial cells with irregular arrangement of their nuclei were present between the well-preserved epithelium. These parts of epithelium showed pseudostratified appearance. The nuclei of superficially localized cells were relatively preserved.

Borders between the cells that are localized next to the basal membrane were incospicuous. Their nuclei were unequal in shape and showed signs of karyolysis, while heterochromatin appeared as scattered particles of a different size.

Except these two permanent cell populations, single small lymphocytes can be found within endometrial epithelium. The lining epithelium invaginated continuously into the simple, mostly low-cuboidal, glandular epithelium containing rare ciliated cells. The presence of a double nucleated cells and mitosis were signs of an intensive proliferation of the glandular epithelium (Figure 3B).

Some animals in this group showed highly cellular and vascularized loose connective tissue of the lamina propria. It contained numerous fibroblasts, discrete ground...
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substance and rare leukocytes, collagen and elastic fibers, while subepithelial area of the stroma showed prominent microvascular capillary bed.

Intensive reorganization of the stroma was revealed in some animals. Stromal cells were more massive with abundant cytoplasm and very large nuclei with prominent eccentrically positioned nucleolus. These changes were more prominent in the areas where epithelial cells showed signs of a secretory activity. Production of intercellular matrix was increased and it contained prominent vascular network and mobile leukocytes. In subepithelial parts of the stroma, polygonally shaped stromal cells with vacuolated cytoplasm were noted. Numerous mobile blood cells and cells with nuclei with different stages of pyknosis were observed in these areas (Figure 3C).

The myometrium was mostly uniform in its appearance with a domination of the helically arranged and interspersed spindle-shaped smooth muscle cells. Also, these cells showed some proliferative activity that was noted by the distance among their nuclei and prominent vascular bed. On the surface of the uterus, serosa was connected to the myometrium with narrow layer of the subserosal connective tissue (Figure 3A).

Control groups (VZ and VM)

In the control group of animals treated with zearalenone vehiculum (VZ) and melatonine vehicle (VM), qualitative histological analysis of the uterus revealed typical organisation of the organ (Figures 4A-B).

5. DISCUSSION

In the control groups of animals treated with either melatonin or zearalenone vehiculum structure of the uterine wall corresponded to the intact uterus (endometrium, myometrium and perimetrium), which was also described by other authors (17-19). The luminal epithelial cells appeared low columnar suggesting the beginning of the proliferative phase, and tall columnar ciliated and secretory cells, specific to the end of the cycle. The lamina propria was cellular and highly vascularized loose connective tissue, that apart from numerous stromal cells, contained lymphocytes, polymorphonuclear leukocytes and macrophages. Myometrium contained spindle-shaped smooth muscle cells, that showed some proliferative activity during the cycle. Superficially positioned serosa was connected with myometrium by a thin layer of subserosal connective tissue (17-19).

Histological analysis of the uterus of the animals treated with melatonin showed that endometrial epithelium was lower compared to the control group of animals and composed mostly of low columnar cells. The epithelium was comprised of cells with kinocilia and numerous, more massive secretory cells. Lamina propria was thinner compared to the control group and composed of a highly vascularized loose connective tissue. Apart from the abundant stromal cells, it contained leukocytes, discrete bundles of collagen and elastic fibers and scarce intercellular amorphous substance. The glands were loosely coiled and their base extended through the basal layer. Also, proliferation areas were noted as discrete pseudostratification of epithelium.

Myometrium was mostly uniform in its appearance. Smooth muscle cells showed some proliferative activity and areas of hypertrophy of myocytes were also noted which contributed to the more massive myometrium when compared to the control group. This histological finding in uterus can be explained by compromised folliculogenesis and reduced production of estrogen, which results in decreased proliferative activity in uterus, primarily in the endometrium. These events can be related with inhibition of the hypothalamus-hypophysis-ovary axis induced by melatonin (20). Both, some abortive secretory activity of the endometrial cells and sporadic hypertrophy of the myometrium can be attributed to the promoting activity of the melatonin on corpora lutea which is also noted by other authors (13, 20, 21).
In the group of animals treated with zearalenone we found qualitative structural changes of the uterus, specifically in the endometrium and myometrium. In the luminal endometrial epithelium areas of metaplasia were revealed. In these areas cells were organized into two layers. Superficially located cells had intensively stained cytoplasm and nucleus and showed mitotic activity, while deeply located cells were larger with mostly spherical, euchromatic and hypertrophic nuclei. Areas of epithelial hypertrophy, which appeared pseudostratified because of irregular alignment of the epithelial cells' nuclei, were also present. Superficially localized nuclei had relatively preserved appearance and structure, while deeply located nuclei were misaligned, less preserved and polymorphic. Among the areas of altered epithelium, preserved epithelium was present. Stroma was wide, hypercellular, especially in its superficial area with sporadic invasion towards the epithelial surface. In its composition most numerous were cells that appeared like fibroblasts and cells with pale cytoplasm and large nucleus. Also, lymphocytes, granulocytes and macrophages were present. The matrix and prominent blood vessels, that sporadically were hyperaemic, indicated the stimulation of stromal structures. Bases of glands were empty and glandular epithelium showed mitotic activity. In the myometrium hypertrophy and hyperplasia were evident with abundant intercellular amorphous matrix and perivascular leukocyte infiltration. In the areas of hypertrophy smooth muscle cells were prominent with vacuolated cytoplasm, while their nuclei were hypertrophic, elongated with small particles of dispersed heterochromatin. In the areas of hyperplasia myocytes were smaller, with more scarce cytoplasm, while their spindle-shaped nuclei were more closely arranged. In the myometrium prominent network of blood vessels was present (22, 23).

In the group of animals administered with zearalenone and melatonin, qualitative histological analysis showed structure of uterus similar to that found in the control group. Areas of preserved epithelium were dominant. Epithelial cells appeared from low to tall columnar and they extend from the basement membrane to the lumen. Luminal epithelium invaginated continuously into the low columnar glandular epithelium of the straight or slightly coiled uterine glands. Lamina propria was moderately cellular and highly vascularized loose connective tissue which contained fibroblasts, ground substance, leukocytes and fibers. Numerous macrophages that were present in the interglandular stroma of the deep functional layer indicated intensive reorganization of the stroma. Areas with insignificant proliferative activity of the myometrium were discrete, while numerous areas of myocytes' hypertrophy contributed to the massiveness of the myometrium compared to the others.

Unlike 'classic' antiestrogens, antiestrogenic activity of the melatonin is not the result of its interference with estradiol binding to the estrogen receptors (24). Abd-Allah et al. (25) found that intraperitoneal application of melatonin in a dose of 0.8mg/kg for 15 days, reduced estrogen receptors for 59% and increased progesterone receptors for 34% in rat's uterus, compared to the control group of animals. In addition to the fact that the melatonin's activity on uterus can be achieved through the activity of prolactin, estrogen and progesterone (26), its direct effect is made through MT1 and MT2 receptors that are widespread in uterus (13, 27, 28).

6. CONCLUSION
Zearalenone led to the alterations of the uterine structures, predominately in the endometrium that were characterized by metaplasia and hypertrophy of the epithelial cells and hypercellularity of the stroma. In the myometrium, zearalenone induced hypertrophy and hyperplasia of the myocytes. Melatonin, when applied together with zearalenone, blocked the adverse effects of the zearalenone.

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