The effect of exogenous melatonin on the histological changes of the ovary induced by Zearalenone

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Abstract
The mycotoxin zearalenone is often found in cereals and animal feeds. The intake of zearalenone through food can result in the hyperestrogenic syndrome, and is related to ovarian structural and functional alterations in mammals. It competitively binds to estrogen receptors and generates oxidative stress. Melatonin is a hormone produced by the pineal gland with a strong effect on reproduction as it inhibits the hypothalamic-pituitary-gonadal axis. It has also direct and indirect antioxidative effects in the ovarian tissues. Our aim was to explore the effect of melatonin on the histological changes of the ovary induced by zearalenone.

Forty female Wistar rats were divided into five equal groups and treated for 28 days according to the following scheme: 1. Zearalenone vehiculum (sunflower oil)- treated group, 2. Melatonin vehiculum- (5% ethanol in Ringer) treated control group, 3. group treated with zearalenone (0.3 mg/kg b.w), 4. group treated with melatonin (10 mg/kg b.w) and 5. group of rats treated with zearalenone (0.3 mg/kg b.w) and melatonin (10 mg/kg b.w).

Zearalenone induced degenerative changes in all developmental forms of ovarian follicles, hypertrophy of stroma with blood vessel dilatation and hyperemia. The concomitant application of melatonin and zearalenone resulted in milder morphological changes of the ovary, especially of preovulative follicles. Melatonin administration prevents the zearalenone-induced structural alterations on an ovary.

Keywords: zearalenone, melatonin, rat, ovary, histology

Introduction
Mycotoxin zearalenone is most frequently found in corn, wheat, barley, oat as well as in some other human and animal food. Mycotoxin zearalenone has estrogenic, uterotrophic and anabolic effects (Hidy et al., 1977; Ožegović and Pepeljnjak, 1995). Taken per os, zearalenone is quickly resorbed from the digestive tract and transported to the liver where it is accumulated and metabolised under the influence of reductase and esterase enzymes; it is mostly eliminated from the body within 4-5 days. Zearalenone residues and derivatives thereof can be found in edible parts of animals that were fed with contaminated fodder even when they appeared clinically healthy. The residues are cancerogenic and their biological effects may be compared to those of diethylstilbestrol and estradiol (JECFA, 2000; Šefer, 2000). Zearalenone’s biological activity can be explained through competition with 17-β-estradiol to specific binding sites on estrogenic receptors and interference with enzymes participating in the metabolism of steroids (El-Makawy et al., 2001; Gaumy et al., 2001). Zearalenone has a relatively low toxicity with acute oral LD 50 values of >2000–20 000 mg/kg b.w. in mice, rats and guinea pigs (Zinedine et al., 2007).

Reproductive system, both male and female, is considered a main target of zearalenone (Li et al., 2015). The intake of zearalenone through food can result in hyperestrogenism in women, which can in turn be related to ovarian and uterine dysfunction (Shier et al., 2001). The studies on the effects of radioactive zearalenone on mice have shown that it was distributed in estrogen-target tissues such as the uterus and ovaries (Appelgren et al., 1982). Previous studies showed that zearalenone and its derivates, even at picomolar levels, had negative effects on chromatin structure stability and viability thus affecting meiosis of oocytes, mitosis of ovarian granulosa cells, causing maturation delay and degeneration (Li et al., 2015; Dai et al., 2016).

Apart from its estrogenic effects, zearalenone also causes the increase of oxidative stress, which represents another explanation for the toxicity of this mycotoxin (Ouanes et al., 2008).
Melatonin is a hormone synthesized mainly by the pineal gland but also in multiple extra-pineal organs including reproductive system (Lan et al., 2018). It acts as a potent natural antioxidant, free-radical scavenger and inhibitor of pro-oxidative enzymes (Tamura et al., 2012; Pandi-Perumal et al., 2006). Also, it influences the reproductive functions by regulation of hypothalamus-pituitary gland axis and interference with estrogen synthesis and receptors (Tamura et al., 2012; Lima et al., 2015). Thus, melatonin regulates the reproductive function via both, oxidative stress reduction and modification of steroidogenesis (Lima et al., 2015; Tanabe et al., 2015; Lan et al., 2018). Melatonin beneficial effects on oocyte maturation have been documented in vitro and in vivo experiments in animals (Lima et al., 2015; Tanabe et al., 2015; Tamura et al., 2012).

Protective effects of different antioxidants against some mycotoxin derivates, in vivo and in vitro (Li et al., 2015; Lan et al., 2018). Options for possible detoxification of zearalenone represent a major interest to researchers.

In the present study, we carried out a histological analysis of the ovaries in Wistar rats treated with zearalenone and melatonin.

### Material and Methods

**Animals.** For the purposes of this experiment, 40 adult female Wistar rats (Rattus norvegicus), reared in standardised laboratory conditions and housed under a controlled photoperiod with a 12/12-hour light/dark cycle. All the procedures were in accordance with the animal protection laws and approved by the local institutional review board. The animals tested were randomly categorised into five equal groups of eight.

**Experimental design.** We measured the weight of all animals on a daily basis with a view to adjust the dosage of melatonin and hence zearalenone in line with possible weight changes. The data obtained were entered in a protocol we prepared for each tested animal. For the purposes of this experiment, we used pure zearalenone (Sigma-Z-2125 EECNo241-864-O) in dry- powdered preparation, which was dissolved in sunflower oil (oleum helianti) before administration. Melatonin (Sigma-Aldrich, St. Louis, MO, USA) used for the purposes of this experiment was dissolved in vehiculum (5% ethanol in Ringer’s solution; 2 mg/mL) prior to administration.

The first group of animals (VM) was administered with melatonin vehiculum on a daily basis. The second group of animals (M) received melatonin intraperitoneally in the amount of 10 mg/kg of body weight once a day. The third group of animals (VZ) was administered with the zearalenone vehiculum, i.e. sunflower oil by gavage, once a day. The fourth group of animals (Z) was administered with zearalenone (0.3 mg/kg b.w) by gavage once a day; immediately after the application of zearalenone, the group was administered with melatonin intraperitoneally (10 mg/kg b.w.), also once a day. The injections were administered between 6 p.m. and 7 p.m., throughout a 28-day period. At the end of the experiment, the animals were euthanized with ether vapours.

**Histological analysis.** After the animals were sacrificed, both ovaries were weighed and the samples of the left ovary were fixed in 10% buffered formalin. Following the customary procedure of paraffin embedding, the tissue blocks were cut in serial cuts, each 5µm thick. For the staining purposes, we used Mayer’s haematoxylin, eosin and Azan. The ovarian qualitative histological analysis was carried out by an optical microscope (ECLIPSE 400) to which a digital camera had been attached and connected to the control unit and the image projection screen. Analysis of the stromal structures, all developmental forms of ovarian follicles, corpora lutea and the vascular network was carried out.

**Statistical analysis.** All our results were expressed as mean ± standard deviation. Statistically significant differences between groups were determined with Student’s t test and ANOVA test. P-values of less than 0.05 were considered statistically significant. Statistical calculations were performed using the SPSS 15 software (version 13.0, SPSS Inc, Chicago, Illinois, USA) and Microsoft Excel (version 11.0, Microsoft Corporation, Redmond, WA, USA).

### Results

**Gross analysis.** The animals’ body weight was measured both at the beginning (W1) and at the end of the experiment (W2). The independent t-test showed no statistically significant differences in mean body weight of animals in VM, M, VZ and ZM groups at the beginning and at the end of the experiment. However, it is notable that the differences of mean body weight of animals administered with zearalenone were statistically significant (Table 1).

The ANOVA multiple comparison test showed that the mean ovary weight (Table 2.) among the animal groups tested were not statistically significant - neither the weights of the left (p=0.286) nor the right ovary (p=0.103).

**Qualitative histological study.** In group M, which was administered with melatonin the germinative epithelium, tunica albuginea, stroma and the ovarian medulla showed normal histological characteristics. Prominent histological features in this group of animals were the relatively numerous pools of primordial follicles, multiple primary follicles but very few follicles in the advanced stages of development (Fig. 1A). Apart from the developing follicles, we also noted various regressive ovarian follicle forms. There were few corpora lutea in regression, significantly smaller in size with clusters of cells displaying involutive changes, separated by conspicuous connective trajectories into which a dilated capillary network was embedded (Fig. 1B).
Qualitative analysis of rats’ ovaries from the zearalenone group (Z) demonstrated changes in all structural parts of the organ. Our key findings were hypertrophy of the germinative epithelium, increased stroma cellularity, dilatation and hyperemia of the blood vessels in the ovarian cortex and medulla as well as degenerative changes in all developmental stages of the ovarian follicles, which could not be attributed to normal atretic changes (Fig. 2A-D). Follicular cells were separated from the theca cells, showing varied stages of apoptosis thus leaving cavities in places of advanced deterioration. With larger cells, due to the presence of numerous vacuoles, a distinct hyperchromasia of the nucleus and inhomogeneity of the cytoplasm was observed. Even though the degenerative changes may be observed in all the lamellae of the follicular walls, they were particularly invasive in the layers along the antrum, while the detritus of these cells could also be found in the follicular fluid, which is inhomogeneous itself. The oocyte in degenerating follicles exhibited inhomogenous cytoplasm and nucleus, and the distribution of the vitellus granules was uneven. Discrete areas of thickened theca capsule and irregularly shaped cells were observed. A well-developed capillary network was embedded between the theca cells. There were very few hemorrhagic corpora lutea. They were structured by predominant granulosa lutein cells, which were surrounded by theca lutein cells indicating their relatively preserved functionality. Among the small groups and threads of lutein cells, there was a profuse network of dilated hyperemic capillaries.

Table 1. Mean body weight of animals at the beginning and at the end of the experiment

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>Weight (g)</th>
<th>Mean</th>
<th>SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VM W1</td>
<td>206.63</td>
<td>2.07</td>
<td></td>
<td>0.312</td>
</tr>
<tr>
<td>W2</td>
<td>210.25</td>
<td>8.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M W1</td>
<td>221.38</td>
<td>4.34</td>
<td></td>
<td>0.516</td>
</tr>
<tr>
<td>W2</td>
<td>218.75</td>
<td>11.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VZ W1</td>
<td>233.50</td>
<td>8.52</td>
<td></td>
<td>0.119</td>
</tr>
<tr>
<td>W2</td>
<td>227.25</td>
<td>10.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z W1</td>
<td>205.13</td>
<td>2.75</td>
<td></td>
<td>0.014*</td>
</tr>
<tr>
<td>W2</td>
<td>211.88</td>
<td>4.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZM W1</td>
<td>205.25</td>
<td>3.54</td>
<td></td>
<td>0.691</td>
</tr>
<tr>
<td>W2</td>
<td>206.00</td>
<td>6.76</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p<0.05; *Standard deviation; VM – melatonin vehiculum control group; M – melatonin-treated group; VZ – zearalenone vehiculum control group; Z – zearalenone-treated group; ZM – zearalenone and melatonin-treated group

Table 2. Mean left and right ovary weight at the end of the experiment

<table>
<thead>
<tr>
<th>Group of animals</th>
<th>Left ovary (g)</th>
<th>Right ovary (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>VM</td>
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<td>17.03</td>
</tr>
<tr>
<td>M</td>
<td>62.75</td>
<td>17.91</td>
</tr>
<tr>
<td>VZ</td>
<td>69.50</td>
<td>8.73</td>
</tr>
<tr>
<td>Z</td>
<td>59.63</td>
<td>4.60</td>
</tr>
<tr>
<td>ZM</td>
<td>51.63</td>
<td>10.13</td>
</tr>
</tbody>
</table>

*Standard deviation; VM – melatonin vehiculum control group; M – melatonin-treated group; VZ – zearalenone vehiculum control group; Z – zearalenone-treated group; ZM – zearalenone and melatonin-treated group

Figure 1. Histological findings in the ovaries of a control group – melatonin. A Pools of primordial follicles surrounded by numerous spindle fibrocytes arranged in different directions (HE, 400x); B Germinative epithelium, tunica albuginea and the cortical area with regressive forms of follicles and corpora lutea (HE, 100x).
In animals (ZM) that were administered with zearalenone following an immediate administration of melatonin, the ovarian germinative epithelium showed uniform characteristics, as did the connective tissue of the tunica albuginea. The cortical stroma showed uniform cellularity, with dominant fibrocytes and an adjacent, delicate network of collagen fibres. The blood vessels in the ovarian cortex and medulla showed no signs of dilatation and hyperemia. The parenchyma consisted of developing and regressive forms of ovarian follicles and corpora lutea (Fig. 3A). In the peripheral region of the ovarian cortex, we noted pools of the intact primordial follicles (Fig. 3B), whereas deeper in the cortex, we observed localised primary, secondary and tertiary ovarian follicles. Only sporadically in the follicles in early stages of development, it was observed that the oocyte cytoplasm was vacuolised and the follicle cell nucleus hyperchromatic. With more mature follicles, what seemed to be dominant was the multi-layered, avascular follicular cell capsule of uniform features and normal lamellarity (Fig. 3C) surrounding the antral cavity filled with the follicular fluid. The attached oocyte had a homogeneous cytoplasm, even distribution of vitellus granules, and its nucleus had a finely dispersed chromatin. The theca cells mostly showed no signs of structural alterations. Only sporadically in some follicles we noted an uneven lamellarity of the granulosa layer (Fig. 3D). Follicular cells in those places displayed irregularities in the form and structure of the nucleus, they were separated from the theca cells, displaying apoptosis, which was particularly intensive in the layers close to the antrum. Cavities remained in places of advanced follicular cell deterioration, while the detritus of these cells was visible in the follicular fluid.

Numerous massive corpora lutea were found in the ovarian cortex. They consist of even more numerous granulosa lutein cells surrounded by a narrow area of theca lutein cells. Among the small groups and straps of lutein cells, there was a stroma with a well-developed capillary network.
Discussion and conclusions

In control groups of rats treated with the melatonin vehiculum (VM) or the zearalenone vehiculum (VZ), qualitative histological analysis of the ovaries showed that the anatomy of all the structures matched that of the intact organ, and was related to the cycle phase (Fig. 4A-B).

Protective action of melatonin on the estrogenic and anabolic effects of mycotoxin zearalenone was studied. When it comes to the anabolic effects of zearalenone, a significant increase (p=0.014) of the rats’ body weight was observed only in the group administered with zearalenone. This is in accordance with previous studies (Babić et al., 1989). An insignificant increase of the animals’ body mass was recorded in the group of rats administered with both, zearalenone and melatonin, which indicates a blockage of the anabolic effect of zearalenone in the presence of melatonin.

The weight of the left ovary in the melatonin vehiculum group was insignificantly increased in comparison to the organ weight of the melatonin group. This can be explained with the inhibitory effect of melatonin on the release of follicle-stimulating hormone (FSH) and consequently on the function of gonades (Kopprisetti et al., 2008). We have also noted a decrease of the ovary weight after the
administration of zearalenone, particularly in comparison with the group that was treated with sunflower oil. The lowest value of the left ovary weight was detected in zearalenone and melatonin group. A similar trend was found for the weight of the right ovary. The finding of reduced ovary weight in the group administered with zearalenone is consistent with the fact that zearalenone and its derivatives suppress gonadotropins with consequent ovarian atrophy (Ruszas et al., 1979). Melatonin changes the ovary weight and the estrous cycle and protects the ovaries from oxidative stress (Chuffa et al., 2011).

In both control groups histologic analysis of the ovaries showed preserved organ structure as noted by other authors (Yin et al., 2002).

The most prominent findings in the ovaries of rats treated only with melatonin were relatively numerous pools of primordial and primary follicles, but very few in secondary and tertiary stages. Only few but massive developmental and regressive forms of corpora lutea were observed. Such histological findings are indicators of the inhibiting effects of melatonin on folliculogenesis (Altun and Ugur-Alunt, 2007; Koppisetti et al., 2008; Leibteseder, 1989). Prominent morphological changes in the ovaries of animals treated only with zearalenone such as ovarian atrophy, follicular atresia and apoptotic changes of granulosa cells, and deterioration of the oocyte in Graafian follicles were also detected by other authors (Minervini et al., 2005; Tiemann et al., 2003; Wasowicz et al., 2005).

In zearalenone-treated group we observed follicular atresia as well as the hypertrophy of stromal components, which was also described in previous studies (Chang et al., 1979; Šahinović et al., 2013).

The effects of zearalenone on the structural components of the ovary can be related to its estrogenic activity, but also to the inhibition of follicle-stimulating hormone secretion, which suppresses the ovarian follicle development and inhibits the ovulation process, while the luteotropic effect of zearalenone causes the retention of the corpus luteum (Gafoor and Trail, 2006; Osweiler, 1996). Following its binding to estrogen receptors, the zearalenone-receptor complex is transported to the nucleus, causing numerous biological and biochemical effects resulting in the RNA, DNA and protein synthesis (Mitterbauer et al., 2003). Also, data obtained from experimental animal models have shown that zearalenone causes oxidative stress by decreasing the activity of antioxidative enzymes and increasing the prooxidative ones (Abbes et al., 2008; Abbes et al., 2009; Hou et al., 2013). Histological analysis of the corpora lutea in the zearalenone group showed that hemorrhagic corpora lutea were decreased in size, while the other forms were relatively preserved. The histological changes of the ovaries in the study of Chang et al. (1979) were more prominent because of the species differences and duration of zearalenone application. Histological analysis of the ovaries of rats administered with zearalenone and melatonin showed that melatonin reduced the alteration of the ovarian structure caused by zearalenone. Melatonin’s protective effect on ovarian structures, especially on the preovulative follicles in conditions of zearalenone application, can be explained by its antioxidative capacities and the inhibition of the hypothalamic-pituitary-gonadal axis (Koppisetti et al., 2008; Lan et al., 2018; Pandi-Perumal et al., 2006). Melatonin’s protective function towards blood vessels whose dilatation is caused by zearalenone, is achieved in the way that it blocks the potassium canals dependent on calcium, which then leads to vasoconstriction (Altun and Ugur-Alunt, 2007), as our qualitative analysis has shown.

Peroral administration of zearalenone led to a significant increase in the body weight of tested animals and caused prominent alterations of folliculogenesis and degenerative changes of all developmental forms of ovarian follicles, hypertrophy of the stroma with a dilatation and hyperemia of blood vessels in the ovarian cortex and medulla. The application of melatonin in zearalenone-treated animals resulted in milder structural alterations, reflecting a blockage of negative effects of zearalenone.

References


Uticaj egzogenog melatonina na histološke promjene jajnika izazvane zearalenonom

Mikotoksin zearalenon se često pronalazi u žitaricama i stočnoj hrani. Unos zearalenona hranom može rezultirati hiperestrogenim sindromom, a povezan je sa strukturnim i funkcionalnim promjenama jajnika kod sisara. Kompetitivno se vezuje za estrogenske receptore i uzrokuje oksidativni stres. Melatonin je hormon pinealne žlijezde koji ima snažne efekte na reprodukciju zbog inhibicije hipotalamo-hipofizno-gonadne osovine. Također, ima direktno i indirektno antioksidativno dejstvo na tkivo jajnika. Cilj ove studije je istražiti efekat melatonina na histološke promjene jajnika inducirane zearalenonom.

Četrdeset ženki Wistar pacova raspoređeno je u pet jednakih grupa i tretirano 28 dana. Doziranje prema grupama je izvedeno na sljedeći način: 1. grupa – vehikulum za zearalenon (suncokretovo ulje), 2. grupa- vehikulum za melatonin (5% etanol u Ringerovoj otopini), 3. grupa - zearalenon (0,3 mg/kg tt.), 4. grupa - melatonin (10 mg/kg tt) i 5. grupa - zearalenon (0,3 mg/kg tt) i melatonin (10 mg/kg tt).

Zearalenon je inducirao degenerativne promjene u svim razvojnim oblicima folikula jajnika, kao i hipertrofiju strome sa hiperemijom i dilatacijom krvnih sudova. Istovremena primjena melatonina i zearalenona rezultirala je blažim morfološkim promjenama u jajnicima, posebno preovulatornim folikulima. Upotreba melatonina prevenira zearalenonom izazvane strukturne promjene na jajnicima.