Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 27 issue 1, 2017, pp 63-67

MASSIVE APOPTOSIS IN THE OVARIAN FOLLICLES, FOLLOWING CONSUMPTION OF CORN CONTAMINATED WITH *FUSARIUM* SP.

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ABSTRACT: Mycotoxins have various effects on human and animal health. Among these, zearalenone is especially toxic for the reproductive system, exerting an estrogenic effect in animals. For the present study, we harvested ovaries from five sows in a pig farm in northwestern Romania. Subsequently, the samples were processed through paraffin technique for histological examination. The ovaries presented numerous follicles in different stages of evolution or involution. None of the antral follicles reached the ovulatory stage, instead they became atretic follicles, undergoing the same physiological processes, but at a higher extent. These findings suggest that consumption of food infested with Fusarium moulds can lead to a significant decrease of the reproductive life of females.

Keywords: apoptosis, atresia, follicle, sow, zearalenone.

INTRODUCTION:

Apoptosis is the genetically regulated cellular death (suicide) responsible for the disposal of cells that have fulfilled the biological functions (Hussein, 2005). Apoptosis is the most important mechanism by which cells are eliminated during the follicular atresia (Sharma and Bhardwaj, 2009) in the ovary. This statement is supported by several studies that assessed the morphological changes in granulosa cells and in the internal theca folliculi in atretic ovarian follicles (Tilly, 1996).

Ovarian follicular atresia is a phenomenon in all mammalian species that allows the consumption of germ cells surplus all through the life (i.e., during the prenatal, neonatal, pre-pubertal, pubertal and post-reproductive life, and during the estrous cycle, pregnancy, and lactation in mammals) (Bhardwaj and Sharma, 2011). Apoptosis acts in the ovarian follicles during fetal and postnatal periods. Some authors state that, during fetal development, apoptosis occurs only in oocytes, whereas in adult animals is frequently detected in granulosa cells of the preantral and antral ovarian follicles (Hussein, 2005).

Morphologically, the apoptosis is characterized by a series of cellular changes such as: cellular atrophy, membranal wrinkling and cytoplasmic/nuclear fragmentation in oocytes (Chaube *et al*., 2005) and granulosa cells of the ovarian follicles (Sharma and Bhardwaj, 2009). Follicular atresia (usually triggered by some physiological signals) can be sometimes induced by other internal or external factors. Among them, there are some secondary metabolites of moulds, named mycotoxins, which have adverse effects on both humans and animals. The most well-known mycotoxins are aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic mycotoxins and ergot alkaloids. Mycotoxins have various acute or chronic effects on humans and animals, their susceptibility being larger or smaller, depending on the species. Mycotoxins' impact includes loss of human and animal lives, increased cost for human and animal care, decrease of the reproductive potential and elimination of the contamined products from human and animal alimentation (Zain, 2011). Mycotoxins are classified by some authors as hepatotoxins, nephrotoxins, immunotoxins, while others divide them in generic groups as teratogen, mutagen, carcinogen and allergen (Zain, 2011).

Among mycotoxins, zearalenone (ZEA) has a an estrogenic activity (Maragos, 2010) and is especially toxic for the reproductive system (Milicevic *et al*., 2010). Zearalenone is a mycotoxin produced by *Fusarium sp.* mould, that use corn, wheat, barley, oats and sorghum as substrates. It possesses an estrogenic activity since it is able to couple with estrogenic receptors, causing functional changes in the reproductive system similarly with the natural estrogens (Gora *et al*., 2004; Wasowicz *et al*., 2005). The susceptibility of species to mycotoxins has been investigated by many researchers, most of them concluding that swines are the most senstive, especially the subjects before puberty (Macri *et al*., 2009; Gupta, 2011).

Mycotoxins produced by Fusarium moulds can cause different infections in humans, including superficial ones, locally invasive or disseminated. The clinical manifestation of fusariosis depends to a large extent on the immune status of the host and the entryway of the mould (Nucci and Anaissie, 2007).

MATERIAL AND METHOD:

The research was conducted in a pig farm in northwestern Romania, which had an effective of 500 fattening pigs and 45 sows for breeding. Corn was the

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majoritary component from the forage administered to animals. Breeding sows showed signs of hyperestrogenism (hipotrepsy, vulvar congestion and swelling, irregular cycles, infertility etc.).

Ovaries were collected from five sows (by performing ovariectomy) to carry out the histopathological investigation. The ovaries were fixed in 10% buffered formalin for 5 days, dehydrated with alcohol, cleared with butyl alcohol (n-butanol) and embedded in paraffin. Harvested ovaries were subsequently sectioned at 5 µm thickness, stained with Goldner's trichrome method and examined under an Olympus BX41 light microscope endowed with an Olympus E 330 photocamera. The obtained images were edited using Adobe Photoshop CS 2.0 program.

RESULTS AND DISCUSSIONS:

The histological specimens from ovaries reveal a special activity, reflected by the existence of numerous follicles in different stages of evolution or involution. Although a large number of ovarian follicles are antral follicles, none of them reach the final ovulatory stage, all ovarian follicles undergo involutive processes (i.e. follicular atresia).

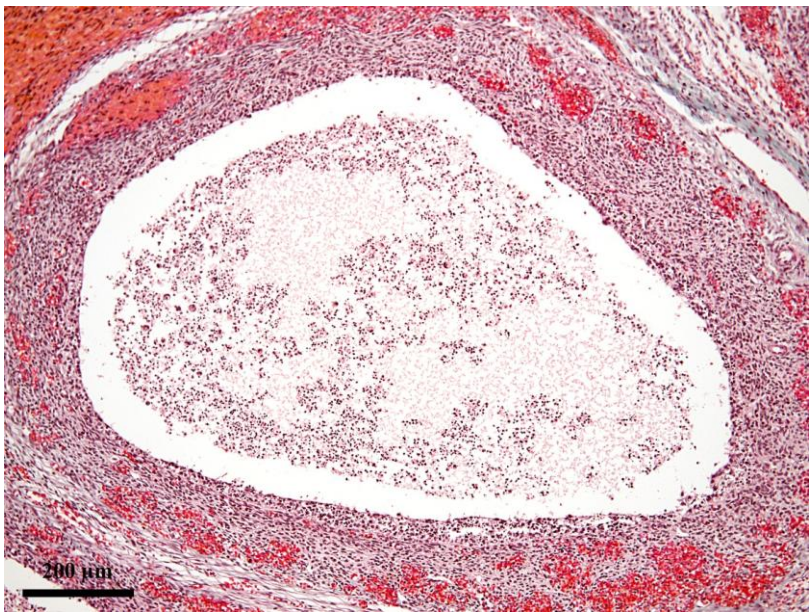

Fig. 1. Cellular debris in the ovarian Graafian follicle (Goldner's trichrome)

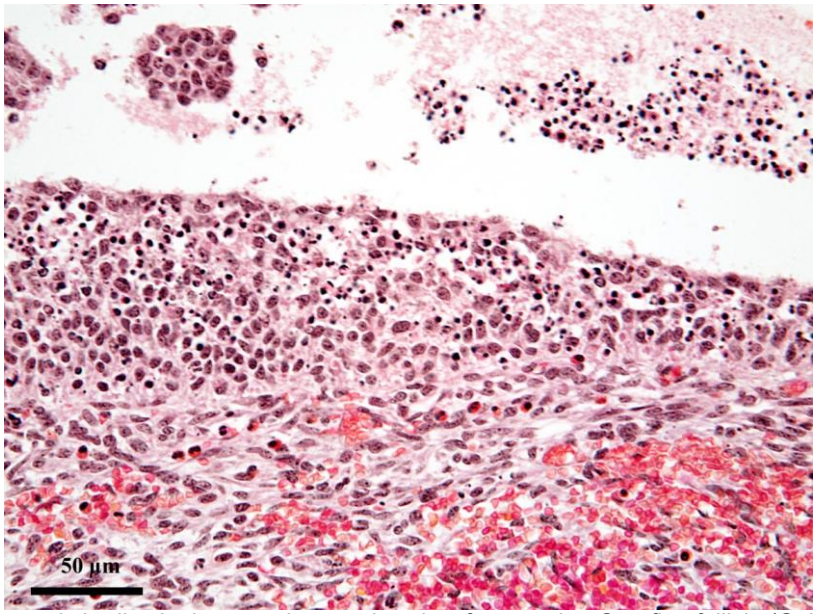

Fig. 2. Numerous apoptotic bodies in the granulosa and cavity of an ovarian Graafian follicle (Goldner's trichrome)

The mechanism of antral follicles atresia in our ovarian samples is similar to the one occurring in physiological apoptosis, except here it unreels at a higher intensity. Accordingly, a large number of follicular granulosa cells undergo apoptosis and the apoptotic bodies separate and accumulate in large quantities in the follicular cavity (Fig. 1). The apoptosis is present in all layers of granulosa cells, but it is more frequent towards the follicular cavity (Fig. 2).

In some microscopical fields there is a significant diminishment of the follicular granulosa cell layers.

Additionally, a complete absence of the granulosa cells has been noticed (aspect frequently associated with a naked basement membrane) (Fig. 3). As a consequence, a large mass made of apoptotic bodies/cellular debris and desquamated cells generate the bulk of the follicular liquid. Several macrophages have been also detected (which have an intense phagocytic activity suggested by numerous cytoplasmic phagosomes), probably arrived here in order to remove the intrafolicular cellular detritus (Fig. 4).

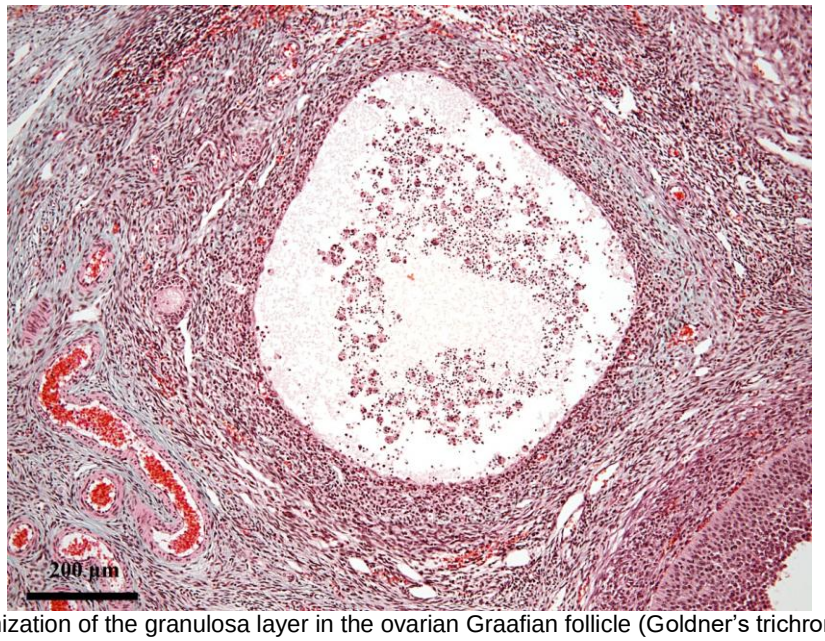

Fig. 3. The disorganization of the granulosa layer in the ovarian Graafian follicle (Goldner's trichrome)

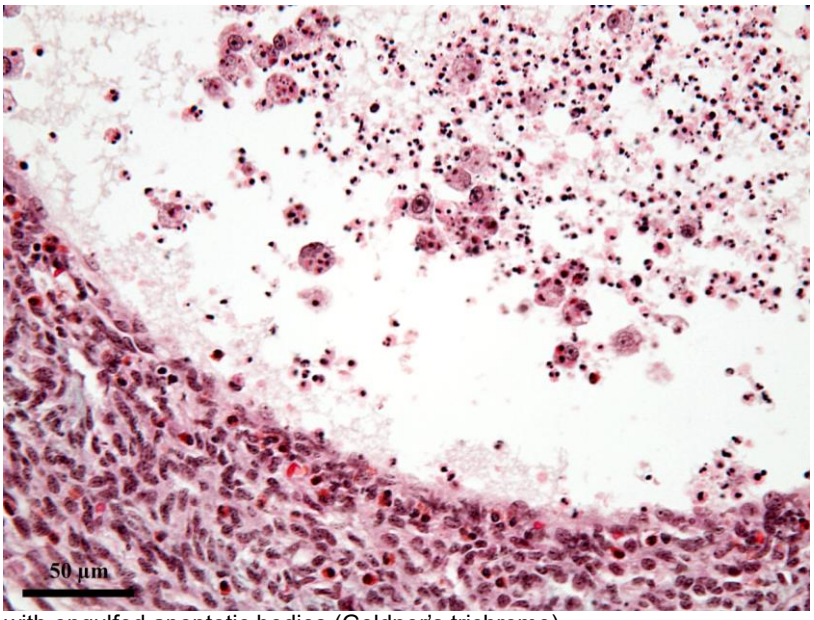

Fig.4. Macrophages with engulfed apoptotic bodies (Goldner's trichrome)

Throughout the process of follicular atresia, the most important mechanism by which follicular cells are eliminated is apoptosis (Sharma and Bhardwaj, 2009). Involutive processes of the antral follicles imply apoptosis of the granulosa cells (Hussein, 2005). Morphological details concerning apoptosis of the ovarian cells were described some time ago by Flamming (1885). The former author wrote about the histological details concerning follicular granulosa cells during degeneration of the antral follicles in the ovary of rabbits. The mechanisms by which granulosa cells are removed have been called chromatolysis (Flamming, 1885 cit. by Kerr *et al*., 1972). The histological details suggested by Flamming (1885) match the morphological characteristics of apoptosis available in the current information (Kerr *et al*., 1972).

Pathological follicular atresia triggered by the action of zearalenone is substantially the same as in the case of physiological atresia. The main difference is the speed that is much higher following zearalenone exposure of sows. Hence, a large number of ovarian follicles from the follicular reserve will be consumed in a relatively short period of time. Consequently, a severe decline of the reserve ovarian follicles (i.e. the ovarian pool) takes place and sows are compromised (at least concerning the reproduction).

The negative action of zearalenone on the reproductive system has been investigated by many researchers. It was found that if its concentration in the diet is high (12 mg/kg diet), following administration during several days (e.g. 10 days), the reproductive performance may be affected (low fertility and ovulation rate) (Zain, 2011). Besides, the peroral administration of the mycotoxin can significantly delay the follicular maturation, ovulation and possibly the luteinization (Huszenicza *et al*., 2000). Zearalenone and its derivates inhibit the oocyte maturation *in vitro* and increase the ratio of oocytes with chromosomal abnormalities; the former effects were dose dependent (Minervini and Dell'Aquila, 2008).

Yang *et al*. (1995) specified several changes in the female genital tract following the consumption of zearalenone in sows. The author detected ovarian follicular atrophy and degeneration, and additional changes in the female genital tract (e.g. mild to severe uterine atrophy, pyometra and endometritis). Similarly, Zielonka *et al*. (2009) suggested that following zearalenone exposure of the female pigs, the ovarian lesions were represented by degradation of the ovarian follicles. In another study, the histological changes in the zearalenone-treated piglets suggested that the ovaries were inflamed and hyperestrogenized by dietary zearalenone, aspect supported by the hyperplasia of the vascular wall in the medulla and hyperemia in blood vessels of the ovary. Additionally, authors detected two primary oocytes in one primary follicle, and numerous primordial follicles in the ovarian cortex (Jiang *et al*., 2010).

Oppositely, Wasowicz *et al*. (2005) reported that zearalenone (20 or 40 µg/kg bodyweight) did not induce apoptosis in swine ovaries. The authors mention that zearalenone does not trigger apoptosis in porcine ovaries, and the inhibition of proliferation must be associated with other mechanisms.

However, some other changes may arise in the female genital tract following zearalenone consumption by sows. Accordingly, Diekman and Long (1989) found that blastocyst's progress was influenced in zearalenone-treated sows that received contaminated feed 7 days after breeding, whereas other reports showed that zearalenone had proliferation effects on uterine cells in gilts (Obremski *et al*., 2003; Zwierzchowski *et al*., 2005).

A number of experimental studies have been conducted in bitches that were exposed to zearalenone. Gajecka *et al*. (2004) proved that the long-lasting intake of food with a low dose of this micotoxin may be the factor which complicates hormonal regulation of the reproduction processes and is the cause of many disorders. Administration of zearalenone in particularly higher doses in pre-pubertal bitches, resulted in hyperestrogenism degeneration and atrophy of ovarian cells and tissues with accompanying edema and blood extravasation, leading to increased 17beta-estradiol concentrations and an insignificant decrease in progesterone levels (Gajecka, 2013a). However, results of the ultra-structural examinations of the ovaries (in the pre-pubertal bitches) revealed that experimental, zearalenone-induced hyperestrogenism enhanced apoptosis and lowered the proliferative ability of follicular cells, which contributed to organelle destruction in pre-pubertal bitches. The changes observed were particularly advanced in animals, which were administered a higher dose of zearalenone (Gajecka, 2013b).

Comparison of zearalenone exposure to the previous daily tolerable intake values, suggests that in many countries, exposure of the population maintains at levels which are considered safe. The situation is different when consuming a large quantity of food susceptible to contamination, or when the contamination is atypically high. For the most part of the world population, no estimates of the exposure were reported, which means that the true extent of ZEA relevance for human health remains still unclear (Maragos, 2010).

CONCLUSION:

This study reveals that after consumption of corn infested with Fusarium moulds, a massive apoptosis of cells in theca granulosa is triggered, in a large number of follicles. All these follicles will eventually be eliminated through atresia in a relatively short period of time. The consequence is the drastic reduction of the follicular pool and the significant decrease of the reproductive life of females. Although the fact that sows are the most sensitive to the action of mycotoxins with an estrogenic action (ZEA) is well known, this does not mean that they do not act similarly in other mammal species and even humans.

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