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Mycotoxin – induced apoptosis in swine kidney epithelial cells

Introduction

Apoptosis is a programmed cell death that requires energy input and the activation of many genes controlled by a variety of proteins primarily of Bcl-2 family. Promoting proteins include, among others: Bax, Bad, Bid, and Bcl-xs, while the inhibiting ones are Bcl-2, Bcl-x1, Bcl-w (Haake, Polakowska, 1993; Ockner, 2001; Elmore, 2007; Stępień et al., 2007). Programmed cell death can be caused by a number of factors such as UV and gamma radiation, cytostatics, bacteria, toxins, reactive oxygen species, oxidative stress or DNA damage (Stępień et al., 2007).

Process of apoptosis occurs in three stages. At first, the process induction signal is generated. In the second stage, caspases activation occurs. Then, during the third stage, phagocytosis of apoptotic bodies by macrophages can be observed (Kopaczewska, Kopaczewski, 2004). A given cell death can be distinguished on a basis of molecular, biochemical and morphological changes. The latter include: condensation of cytoplasm and chromatin, shrinkage and nucleus fragmentation, as well as formation of blisters and apoptotic bodies. There is also the release of cytochrome *c* from mitochondria, decrease in mitochondrial potential, cell dehydration, increase in calcium ion concentration, activation of serine proteases and caspases, loss of asymmetry in cell membrane phospholipids distribution, as well as degradation of actin filaments and DNA.

Apoptosis can occur in different ways, depending on the pathway. There are external, internal, pseudo-receptor, sphingomyelin-ceramide and stress-induced types distinguished. In the first type, the pathway is induced by combining the ligand with a membrane receptor, resulting in death signal transmission and caspase cascade activation. Contradictory to that, the internal pathway, also known as mitochondrial, is invoked by, among others, the influence of reactive oxygen species, oxidative stress or DNA damage. This leads to the opening of mitochondrial channels and

cytochrome *c* release. Presence of pseudo-receptor pathway was observed in the natural killer (NK) cells and cytotoxic T lymphocytes, while sphingomyelin-ceramide pathway may be activated by viral infections or ionizing radiation. The last apoptosis pathway (stress-induced) was discovered in 2000 (Nakagawa et al., 2000). It arises from the accumulation of incorrectly folded proteins in the endoplasmic reticulum (Kerr et al., 1972; Haake, Polakowska, 1993; Ockner, 2001; Fadeel, Orrenius, 2005; Elmore, 2007).

Apoptosis is an important physiological process occurring in the postnatal period and during embryogenesis. Disturbance of this process can lead to the occurrence of such diseases as cancer, Parkinson's, Huntington's or Alzheimer's diseases, heart attack or brain stroke (Elmore, 2007).

Process of apoptosis can be tested in many various ways (Darzynkiewicz et al., 1997; Smolewski, Darzynkiewicz, 2003; Elmore, 2007). There are cytometric and non-cytometric methods. The latter include the cell morphology analysis using dyes such as hematoxylin and eosin or fluorochromes, e.g. DAPI. Changes are assessed by means of light, fluorescence and electron microscope. One of the apoptosis symptoms is DNA fragmentation that can be analyzed using agarose gel electrophoresis and by comet assay. The cytometric methods include: analysis of laser light scattering by apoptotic cells, study of cell membrane permeability disturbances using dyes such as: trypan blue, ethidium bromide, 7-AAD (7-aminoactinomycin), as well as study of conformational changes in cell membrane using annexin V. The study frequently analyses the phenomena associated with the participation of mitochondria in programmed cell death, e.g. mitochondrial potential drop using dyes (rhodamine 123), translocation of Bax to the mitochondria or the release of cytochrome *c* from mitochondria into cytoplasm by means of Western blotting using antibodies conjugated with a fluorochrome (Darzynkiewicz et al., 1997; Smolewski, Darzynkiewicz, 2003).

Mycotoxins are secondary metabolites produced by a wide range of different molds including *Aspergillus*, *Penicillium*, *Fusarium* and *Stachybotrys* spp. Biosynthesis of these compounds can take place during storage and processing of raw materials and during plant growth as well. Mycotoxins are a potential threat to the health of animals and humans. They enter the organism not only by ingestion, but also through inhalation of contaminated air. Secondary metabolites of molds can cause mycotoxicosis, which causes damage to internal organs and skin tissues (Grajewski, 2006). The number of adverse effects of mycotoxins on an animal organism have been shown, e.g. dermotoxic, estrogenic, hepatotoxic, immunotoxic, carcinogenic, mutagenic, teratogenic and toxic actions to the hematopoietic system (Wróbel, 2014).

The most common mycotoxins include: aflatoxins, fumonisins, ochratoxin A, trichothecenes (T2, HT2, deoxynivalenol) and zearalenone. Trichothecenes are secondary metabolites of *Fusarium* that occur in cereals and cereal products. They have

dermatoxic and hemorrhagic action (Chen et al., 2008; Liu et al., 2014). Aflatoxins are primarily synthesized by *Aspergillus* and are being found in peanuts, spices, dried fruits, cotton pulp and maize grain (Twarużek et al., 2013). Aflatoxin B_1 is the most toxic metabolite. The milk can contain aflatoxin M_1 (Zastempowska et al., 2016). Aflatoxins are potent poisons having teratogenic, mutagenic and carcinogenic properties (Wróbel, 2014; Liu et al., 2015a; Peng et al., 2016). There is also ochratoxin A synthesized by *Penicillium* and *Aspergillus*, and occurs in cereal grain, dried fruits, coffee, spices, animal-origin food and wine (Kosicki et al., 2016). It causes irreversible damage to the nephrons and reveals carcinogenic and immunotoxic action (Grajewski et al., 2007; Zhang et al., 2009; Chopra et al., 2010; Liu et al., 2015b).

The aim of this study was to analyze the impact of mycotoxins: aflatoxin B1, ochratoxin A and toxin T2 on the cells of swine kidney (SK) epithelial cell line and their apoptosis.

Materials and methods

Cell culture and treatment conditions

The SK (swine kidney epithelial) cell line derived from the Department of Physiology and Toxicology of the Kazimierz University of Bydgoszcz were the material for research. The cells were cultured in MEM (Minimum Essential Medium Eagle) supplemented with antibiotics (penicillin and streptomycin) and 5% fetal calf serum. Cultures (adjusted to 4×10^5 cells/ml) were grown for 24 h at 37°C, at the access for 5% CO_2 and at 95% humidity. Then cells were incubated for 24 h with mycotoxins in concentrations chosen experimentally and based on literature: T2 toxin at 2.5 μM and 25 μM dose, aflatoxin B_1 at concentrations of 10 μM and 30 μM and ochratoxin A of 50 μM and 80 μM. Cell line SK cultured in MEM supplemented with antibiotics and fetal calf serum constituted the control.

Analysis of apoptosis

Flow cytometer Muse Cell Analyzer, as well as *Muse Annexin V & Dead Cell* Kit (Merck) were used for evaluation of apoptosis. The cytometer uses laser-based fluorescent detection and microcapillary technology to deliver quantitative cell analysis. Cells used in the culture were separated from the base using 0.25% trypsin and then incubated for 20 min with Muse Annexin V & Dead Cell Kit in accordance with the attached protocol. Muse Annexin V & Dead Cell Kit contains fluorescently labelled annexin V, which, as the coagulant, binds with negatively charged phospholipids such as phosphatidylserine moving outside the plasma membrane upon the onset of apoptosis. This procedure allows to detect cells in various stages of apoptosis: live cells, dead cells, early and late apoptotic cells.

Results and discussion

Studies have shown sensitivity of SK cell line on mycotoxins, namely T2 toxin, aflatoxin B_1 and ochratoxin A (Tab. 1, Fig. 1–2). The data indicates a relationship between the dose of mycotoxins and the occurrence of apoptosis. The highest percentage of apoptotic cells was observed in those treated with aflatoxin B_1 at a dose of 30 μM, and ochratoxin A at both concentrations. There were no clear differences in the effect of ochratoxin A at both concentrations, i.e. 50 μM and 80 μM (Tab. 1, Fig. 1–2). Mycotoxin the least interacting on SK cell line turned out to be T2 toxin.

Tab. 1. Influence of mycotoxins on the presence of apoptosis in the swine kidney epithelial cell line

Toxin (dose)	Live [%]	Early apoptotic [%]	Late apoptotic [%]	Total apoptotic [%]	Dead [%]
Control	93.90	5.35	0.15	5.50	0.60
T2 (2,5 μM)	93.04	3.36	3.53	6.90	0.06
T2 (25 μM)	73.55	15.10	11.25	26.35	0.10
aflatoxin B_1 (10 μM)	85.90	12.80	0.70	13.50	0.60
aflatoxin B_1 (30 μM)	55.05	26.05	14.55	40.60	4.35
ochratoxin A (50 μM)	31.49	64.77	3.74	68.51	0
ochratoxin A (80 μM)	39.59	52.47	7.94	60.41	0

Mycotoxins such as trichothecenes (T2 toxin, HT2, deoxynivalenol), aflatoxins, ochratoxin A, fumonisins and zearalenone can lead to the cell death. Most often, apoptosis and necrosis can be observed. Apoptosis is a programmed cell death and, in contrast to necrosis, is the active process that needs some energy input and gene activation (Majno, Joris, 1995; Trump et al., 1997; Stępień et al., 2007). During this process, chromatin condensation, DNA fragmentation, formation of apoptotic bodies and phagocytosis take place. Programmed cell death is a physiological process occurring in the postnatal period and during embryogenesis. Disorders during its course can lead to the occurrence of many diseases (Elmore, 2007). In contrast to apoptosis, necrosis is a pathological process affecting the cell groups. It brings cells to swell, causes disintegration of organelles and induces inflammation (Majno, Joris, 1995; Trump et al., 1997). These studies have confirmed the ability of selected mycotoxins to induce apoptosis.

Our study showed that T2 toxin proved to be the least toxic mycotoxin (at least at the concentrations used in the experiment). The T2 toxin is derived from trichothecenes group. The exact mechanism of this substance action is not known. It is believed that T2 toxin is an inhibitor of protein synthesis, induces lipid peroxidation and

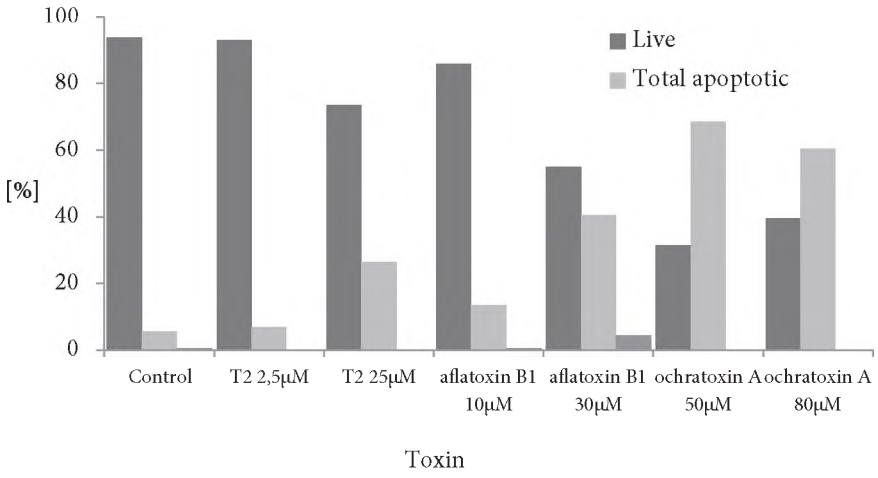

Fig. 1. Influence of mycotoxins on the presence of apoptosis in the swine kidney epithelial cell line

inhibits synthesis of DNA and RNA (Doi et al., 2008). It is well known that T2 toxin induces apoptosis (Shinozuka et al., 1997; Li et al., 1997; Wang et al., 2012), but the research of molecular mechanism of this process is still unknown.

Scientists suspect that apoptosis can probably occur through the extrinsic pathway with the use of membrane receptors and their ligand (Chen et al., 2008). The death receptors include TNF (tumor necrosis factor), while TNF-alpha or FasL as its ligand (Haake, Polakowska, 1993; Ockner, 2001; Fadeel, Orrenius, 2005; Elmore, 2007). Combination of the relevant molecules activates the caspase cascade, which in consequence leads to death. Chen et al. (2008) studied the effects of T2 on human chondrocytes. Their results showed the increment of Fas, p53, Bax, caspase-3 and a decrease of Bcl-2 and, therefore, indicated the presence of apoptosis. It is believed that it might have occurred with the use of an external pathway. The researchers suggest that the T2 toxin can cause Kashin-Beck disease, which causes damage to the joints (Chen et al., 2008). When administered orally, parenterally and in contact with the skin, T2 induces apoptosis in thymocytes, in the thymus lymphocytes, hematopoietic cells, epithelial cells of the intestinal crypts, hepatocytes and keratinocytes in animals. This process was also noted in the red and white pulp of the spleen, and in Peyer's patches as well (Chen et al., 2008; Doi et al., 2008; Liu et al., 2014). It is worth mentioning that the toxin penetrates the placenta and affects the fetus, among others, by damaging its brain (Doi et al., 2008). In addition to T2, ochratoxin A at concentrations of 50 μM and 80 μM was also tested in the present study. It has been shown that both doses caused apoptosis in about 60% of the cells. Ochratoxin A is classified as a human carcinogen. Liu et al. (2015b) studied the effect of the substance on Het-1A cells (cancer of the esophagus).

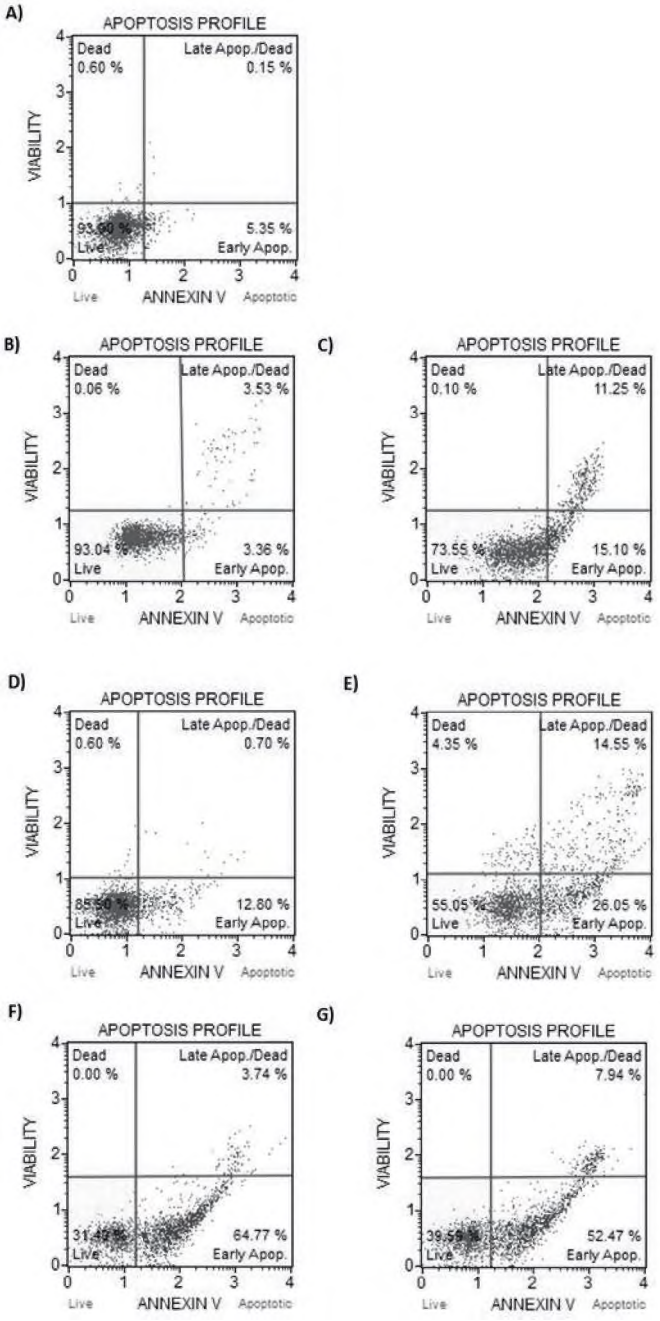

Fig. 2. The occurrence of apoptosis in the swine kidney (SK) epithelial cells after treatment with mycotoxins at different concentrations: a – control, B – 2.5 μM T2, C – 25 μM T2, D – 10 μM aflatoxin B_1, E – 30 μM aflatoxin B_1, F – 50 μM ochratoxin A, G – 80 μM ochratoxin A. The cells have been divided into four distinct populations: live (the lower left square), early apoptotic (right lower square), late apoptotic (right upper square) and dead (left upper square). Results have been expressed as percentages

The DNA strand breaks were observed along with chromosomal aberrations, and arrest of the cell cycle in the G2 phase. Moreover, there was an increase of caspase-3, which suggests the occurrence of apoptosis in Het-1A cells. Elevated activation of caspase-3 was also observed in a study upon the immunotoxicity of ochratoxin a in cells of H9 line (human T lymphocytes).

In addition, changes were seen at the cell morphology level (condensed cytoplasm, chromatin condensation, apoptotic bodies, swollen mitochondria), the increase in TNF-alpha, and a decrease of IL-2. It is suggested that necrosis has the priority over apoptosis at higher concentrations of ochratoxin A. The researchers turned their attention to the possibility of the apoptosis occurrence through the mitochondrial pathway, otherwise known as internal pathway (Darif et al., 2016). Mitochondrial channels are opened under the influence of stress-inducing factors and cytochrome *c* is released into the cytoplasm. This compound binds to cytoplasmic factor Apaf 1 and inactive caspase-9. Then, activation of caspase-9 and executive caspases takes place, which leads to cell death (Elmore, 2007; Stępień et al., 2007; Darif et al., 2016). Ochratoxin A has nephrotoxic, hepatotoxic and immunotoxic action. Other studies have shown the dose-dependent manner increase in cytotoxicity on neural/nerve cells (Zhang et al., 2009).

In the present experiment, we demonstrated a dose-dependent manner effect of aflatoxin B_1 on the SK line cells. a dose of 10 μM resulted in an increase of apoptotic cells by 8%, while 30μM dose by 35% in comparison to the control version. Aflatoxin B_1 interferes with the porcine oocyte maturation by inducing the oxidative stress, and also causes the occurrence of excessive autophagy and apoptosis (Liu et al., 2015a). Both apoptosis and autophagy are a type of programmed cell death (Stępień et al., 2007). Autophagy is present in all eukaryotic cells and is activated in a response to a shortage of development-inducing nutrients, as well as damage due to toxins. It is characterized by a lack of the nucleus fragmentation, degradation of the Golgi apparatus or the endoplasmic reticulum, and partial chromatin condensation. Peng et al. (2016) studied the role of mitochondria, death receptors and endoplasmic reticulum in the toxin-invoked pathways of thymocytes apoptosis induced by aflatoxin B_1. The study revealed the importance of external and internal pathways in the occurrence of apoptosis. It was also noted that the mycotoxin induces apoptosis, but also necrosis in human lymphocytes (Al-Hammadi et al., 2014).

Conclusions

The studies presented in this article have shown the impact of mycotoxins such as T2 toxin, aflatoxin B_1 and ochratoxin A on the SK line cells. The scientific literature confirms the toxic effects of mycotoxins on a variety of cell lines of animal, as well as human organisms. Unfortunately, we still can not explain the molecular mechanisms

of apoptosis occurrence in cells. It is also possible the existence of other types of cell death, e.g. necrosis, mitotic catastrophe, or autophagy as the reaction to fungal toxins.

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Abstract

Apoptosis, as the programmed cell death, plays a significant role in proper functioning of an organism, both in the postnatal period and during embryogenesis. Disturbances in this process can lead to the occurrence of several dysfunctions, e.g. cancer, stroke, Alzheimer's disease and others. Apoptosis can be triggered by factors such as oxidative stress, free radicals, UV radiation and cytotoxic drugs, but also mycotoxins, e.g. aflatoxins, ochratoxin A and trichothecenes. These toxins are produced primarily by fungi of *Aspergillus*, *Penicillium*, *Fusarium* and *Stachybotrys* genera. The aim of the study was to investigate the effect of mycotoxins on the occurrence of apoptosis in the swine kidney (SK) epithelial cells. For this purpose, trichothecene T2 toxin was used at a concentration of 2.5 μM and 25 μM, aflatoxin B_1 at a dose of 10 μM and 30 μM, and ochratoxin A concentrations of 50 μM and 80 μM. The results were assessed using flow cytometer Muse Cell Analyzer (Merck). Studies have shown high sensitivity of the cell line SK on mycotoxins. Apoptosis was caused by all kinds of toxins and depended on the dose of ex-

amined substance. T2 toxin at a concentration of 2.5 μM caused apoptosis in 6.9% of the cells, whereas at a concentration of 25 μM in 26.35% of the cells. Aflatoxin B_1 used at concentrations of 10 μM and 30 μM caused apoptosis in 13.5% and 40.6% of the cells, respectively. The use of ochratoxin A in concentrations of 50 μM and 80 μM caused the occurrence of apoptosis respectively in 68.51% and 60.41% of the cells.

Key words: aflatoxins, apoptosis, mycotoxins, ochratoxin A, swine kidney cell line, T2 toxin

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Apoptoza w komórkach nabłonkowych nerki świni indukowana mykotoksynami

Streszczenie

Istotną rolę w prawidłowym funkcjonowaniu organizmu odgrywa apoptoza jako programowa śmierć komórki, zarówno w okresie poporodowym, jak i podczas embriogenezy. Zaburzenia tego procesu mogą prowadzić do wystąpienia różnych schorzeń, np. raka, udaru mózgu, choroby Alzheimera i innych. Apoptoza może być efektem takich czynników jak: stres oksydacyjny, wolne rodniki, promieniowanie UV, leki cytotoksyczne, w tym również mykotoksyny. Do najczęstszych mykotoksyn należą: aflatoksyny, ochratoksyna A i trichoteceny. Są one produkowane głównie przez grzyby *Aspergillus*, *Penicillium*, *Fusarium* oraz *Stachybotrys*. Celem pracy było zbadanie wpływu mykotoksyn na występowanie apoptozy w komórkach nabłonkowych nerki świni (SK). W eksperymencie zastosowano toksynę T2 w stężeniu 2,5 μM i 25 μM, aflatoksynę B_1 w dawce 10 μM i 30 μM oraz ochratoksynę A w stężeniu 50 μM i 80 μM. Wyniki oceniano za pomocą cytometru przepływowego Muse Cell Analyzer (firmy Merck). Badania wykazały wysoką wrażliwość linii komórkowej SK na mykotoksyny. Apoptoza była wywoływana przez wszystkie rodzaje toksyn i była zależna od dawki badanej substancji. Toksyna T2 w stężeniu 2,5 μM powodowały apoptozę u 6,9% komórek, podczas gdy w stężeniu 25 μM stwierdzono jej obecność u 26,35% komórek. Aflatoksyna B_1 stosowane w stężeniach 10 μM i 30 μM powodowały apoptozę komórek odpowiednio u 13,5% i 40,6% komórek. Zastosowanie ochratoksyny A w stężeniu 50 μM i 80 μM powodowało wystąpienie apoptozy u 68,51% oraz 60,41% komórek.

Słowa kluczowe: aflatoksyny, apoptoza, mykotoksyny, ochratoksyna A, linia komórkowa SK, T2 toksyna

Information on the authors

Agnieszka Chrustek
She is interested in the identification and analysis of pathways leading to the occurrence of cell death (apoptosis, necrosis, mitotic catastrophe). The research are carried out using light, fluorescence and electron microscopy, flow cytometry and Western blot method.

Magdalena Twarużek
Her main research interests revolve around the following thematic groups: the occurrence of mold and mycotoxins in food, feed and environment of humans and animals, the effect of molds and mycotoxins on human and animal health and the use of *in vitro* culture in the evaluation of contamination.

Ewa Zastempowska
The subject of her scientific interests are microorganisms that are causative agents of mammary gland inflammation (mastitis) in cows. She is also interested in problems related to virulence, drug resistance and cytotoxicity of the pathogenic microorganisms isolated from the animal milk and the presence of mycotoxins in milk produced by molds as well.

Jan Grajewski
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