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Comparative effect of ochratoxin A on inflammation and oxidative stress parameters in gut and kidney of piglets

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ABSTRACT

Ochratoxin A (OTA) is a secondary metabolite produced by fungi of *Aspergillus* and *Penicillium* genra. OTA is mainly nephrotoxic but can also cause hepatotoxicity, mutagenicity, teratogenicity, neurotoxicity and immunotoxicity. As recent studies have highlighted the close relationship between gastrointestinal tract and kidney, as principal organs involved in absorption and respective excretion of xenobiotics, the aim of the present study was to analyze the effect of a *subchronic* exposure (30 days) to 0.05 mg/kg OTA on immune response and oxidative stress parameters at the level of intestine and kidney of young swine. The experiment was realised on twelve crossbred weaned piglets randomly allotted to both control group or toxin group fed 0.050 mg OTA/kg feed. Our results have shown that a *subchronic* intoxication with a low dose of OTA for 30 days affected the immune response and the anti-oxidant self-defense at gut and kidney level. The gene expression of both markers of signaling pathways involved in inflammation and inflammatory cytokines were affected in a much higher extent in the gut than in the kidney Of OTA intoxicated piglets.

1. Introduction

Ochratoxins are a group of secondary metabolites produced by *Aspergillus* and *Penicillium* sp and ochratoxin A (OTA) is the most toxic molecule in this group (el Khoury and Atoui, 2010).

The contamination with OTA of the food and feed commodities is very heterogeneous due to climate or storage conditions (Malir et al., 2016). The contamination with high concentration of toxin is quite rare, the food and feed being usually contaminated with *concentrations of OTA below the EU guidance value* (Marin et al., 2016).

Ochratoxin A is nephrotoxic in both human and animal species studied (Malir et al., 2016). Kidney is the primary target organ, but as shown by *in vivo* animal studies, ochratoxin A can also cause hepatotoxicity, mutagenicity, teratogenicity, neurotoxicity and immunotoxicity (Kuiper-Goodman and Scott, 1989; Marin et al., 2016). For example, human OTA exposure was associated with different kidney pathologies, as Balkan endemic nephropathy, chronic interstitial nephritis and karyomegalic interstitial nephritis (Simon et al., 1996). In term of immunotoxicity, it was shown that OTA reduce cell-mediated immunity of pigs, delayed response to immunization, and increased susceptibility to infection (Stoev et al., 2000). Experimental studies indicated that OTA modulates the immune response even at levels far below the toxicity threshold (Marin and Taranu, 2015) and it was suggested that a network of interacting epigenetic mechanisms, including protein synthesis inhibition, oxidative stress and the activation of specific cell signalling pathways is responsible for OTA immunotoxicity and carcinogenicity (Marin-Kuan et al., 2008).

Pigs are generally considered to be the most sensitive species to the nephrotoxicity induced by OTA and for this reason, the establishment of the provisional tolerable weekly intake for humans had been based on nephrotoxicity studies in pigs (Walker and Larsen, 2005). Exposure to low levels of ochratoxin can decreased pig growth rate and feed efficiency with or without the reduction of the feed consumption (Lippold et al., 1992; Malagutti et al., 2005). These effects were accompanied by hyperproteinemia and increase of serum urea and creatinine, associated with alteration of kidney functions (Battacone et al., 2010; Lippold et al., 1992).

Following oral ingestion, OTA is rapidly absorbed and reaches the systemic circulation, where it is bound in high percentage (99%) to plasma proteins (Ringot et al., 2006). Animal studies have shown that the extent of absorption varies between 40% in chickens and

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66% in pigs (Galtier et al., 1981). *Intestinal cells are the first cells to be exposed to mycotoxins, and often at higher concentrations than other tissues* (Grenier and Applegate, 2013). After the absorption, OTA is principally metabolized in intestine, liver and kidney (Galtier, 1991; Wu et al., 2011). OTA alters intestinal barrier and absorption functions, thus facilitating the translocation of the toxin from gut lumen into the bloodstream (Maresca et al., 2001). Both urine and feces are important excretory pathways for the toxin (Hope and Hope, 2012). For example, OTA and its metabolite Ochratoxin α (OT-alpha) were detected in urine and feces of pigs fed daily with a mixture of OTA and OTB (0.38 and 0.13 mg/kg body weight) for 8 days during early pregnancy (Patterson et al., 1976). The elimination half-lives of OTA in Wistar rats and pigs were reported to be 5 and 6 days, respectively (Dietrich et al., 2005).

Recent studies have highlighted the close relationship between gastrointestinal tract and kidney (frequently referred as the kidney - gut axis) as principal organs involved in absorption and respective excretion of xenobiotics. For example, in humans, chronic kidney disease was associated with the damage of the intestinal epithelial barrier and quantitative/qualitative modifications of the intestinal microbiota (Sabatino et al., 2015). The OTA translocation together with the reabsorption of OTA represent very important toxicological mechanisms which contribute to the long half-life of the mycotoxin in the body (Kuiper-Goodman and Scott, 1989). In this context, the aim of the present study was to compare the effect of a low level of OTA (0.05 mg/kg feed) on kidney and gut (duodenum and colon) by the assessment of some immune response and oxidative stress parameters in pigs. This chosen concentration of OTA represents the guidance values established by the European Commission (EC 576/2006) for OTA in complementary and complete feeding stuffs for pigs.

2. Material and methods

2.1. Reagents

All chemicals, immunological reagents and media components were purchased from Sigma (Sigma-Aldrich, Steinheim, Germany) unless otherwise stated.

2.2. Animals and treatments

Twelve, crossbred weaned piglets (TOPIGS-40), 4-week-old, *females,* with an initial average body weight of 9.83 ± 0.5 kg were exposed for a short period (30 days) to a low dietary OTA contamination. Piglets were individually identified by ear tag, housed in two pens (3 animals/pen; six animals per group) and randomly assigned to either a control group (maize-soybean basal diet without mycotoxin) or OTA contaminated group (basal diet contaminated with 0.05 mg/kg feed) as described by (Marin et al., 2016). At the end of the experiment (day 30), animals were slaughtered by exsanguination in an EU-licenced abattoir according with the EU Council directive 2010/63/CE and samples of kidney, duodenum and colon were taken on ice and stored at −80 °C until the assessment of the immune and stress oxidative parameters.

Animals were raised in agreement with the Romanian Law 43/2014 for handling and protection of animals used for experimental purposes and the EU Council Directive 98/58/EC concerning the protection of farmed animals. The study protocol was accepted by the Ethical Committee of the National Research-Development Institute for Animal Nutrition and Biology, Balotesti, Romania (Ethical Committee no. 52/2014).

2.3. Mycotoxins analyses

The content of OTA was analysed by high performance liquid chromatography (HPLC) with fluorescence detection after clean-up with an immune-affinity column (Ochraprep, R-BIOPHARM). The concentration of OTA was 2.52 ppb in the control feed and 49.62 ppb in the OTA contaminated feed. Other mycotoxins concentration (DON, ZEN, FB1, FB2, T-2, HT-2, DAS, AFB1, AFB2, AFG1, AFG 2) were below the limit of detection.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR) and data analysis

Tissue samples were taken from duodenum, colon and kidney and stored at −80 °C until RNA extraction. Tissue samples were homogenized in liquid nitrogen and total RNAs were extracted as already described (Marin et al., 2016). In order to quantify the expression levels of selected genes, equal amounts of cDNA were synthesized using 1 μg of purified RNA and M-MuLV reverse transcriptase (Fermentas, Thermo Fischer Scientific, USA), as well as oligo(dT) primers (Fermentas, Thermo Fischer Scientific, USA) as already described (Taranu et al., 2015). Fluorescent real-time PCR was used to evaluate the expression of: p38 mitogen-activated protein kinases (p38); nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), tumor necrosis factor (TNF alpha), interleukin beta (IL-1 beta), interleukin 6 (IL-6), interleukin 12 (IL-12), interferon gamma (IFN gamma), interleukin 8 (IL-8), interleukin 17A (IL-17A), interleukin 18 (IL-18), interleukin 4 (IL-4), interleukin 10 (IL-10), cyclooxygenase (COX-2), inducible nitric oxid synthase (iNOS), constitutive nitric oxide synthase (eNOS), hemoxygenase (HO-1), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), catalase (CAT), superoxide dismutase (SOD 1), glutathione peroxidase (GPx).

Synthesized cDNA was diluted 1:50 with nuclease-free water and used for the qRT-PCR together with Maxima SYBR Green/Fluorescein qPCR Master Mix 2X (Fermentas, Thermo Fischer Scientific, USA) and 0.3 μM of both forward and reverse primers. Gene-specific primer pairs obtained from Eurogentec (San Diego, USA) were found in the literature or designed using Primer3 and BLAST (Marin et al. 2013, 2016; Pistol et al., 2014). Thermal cycling was carried out with a Rotor Gene-Q Pure Detection (QIAGEN, Hilden, Germany) using the following conditions: 50 °C, 2 min; 95 °C, 10 min; and 40 cycles at 95 °C, 15 s; 60 °C, 1 min; 72 °C, 30 s and final elongation step at 72 °C for 10 min). Each gene was measured in triplicate and the formation of single PCR products was confirmed using melting curves. Negative controls, which consisted of all of the components of the qPCR mix except cDNA, were used for all primers. The relative quantification of gene expression changes was recorded after normalizing for the average of two reference genes: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin gene expression computed by using the $2^{(-\Delta\Delta\ CT)}$ method (Livak and Schmittgen, 2001) and data were expressed as relative fold increase or decrease from control weaned piglets.

2.5. Enzyme activity assessment

200 mg of frozen kidney, duodenum and colon samples were homogenised in phosphate buffer 50 mM, pH7 and centrifuged for 15 min at 4 °C and 1000 g. Supernatants were used as already described for assessment of the activity of glutathione peroxidase, catalase and superoxide dismutase (Marin et al., 2016).