STUDIES CONCERNING THE NEPHROTOXIC EFFECTS OF OCHRATOXIN A USING PIG AS A MODEL

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Abstract

The Balkan endemic nephropathy (BEN) is a chronic nephropathy described in some regions from the Balkan Peninsula and in Romania. Ochratoxin A is considered to be one of the factors involved in BEN triggering. Ochratoxins are secondary metabolites produced by fungus and it was shown that OTA is principally nephrotoxic but also genotoxic or immunotoxic. The aim of the present paper was to investigate the nephrotoxic effect of a low dose of OTA (50ppb) administrated to weanling pig.

Twelve weanling piglets were fed a corn-soybean meal basal diet for 33 days and randomly assigned to either a control or OTA group. The following biochemical markers were assessed in plasma of weanling piglets: urea, creatinine, phosphorus, calcium, protein and albumin. The expression of genes involved in inflammation: genes for cytokines (IL-1 beta, TNF alpha, IL-8, IL-4) or for other markers (p38, Nf-KB, iNOS, Cox2) were assessed in kidney by real time PCR. The expression of cytokines: IFN gamma, IL-4, IL-6, TNF alpha, IL-1, IL-10 were assessed by ELISA. Administration of 50μg/kg OTA to the weanling piglets has no effect on urea, creatinine, phosphorus or calcium concentration, but induced a significant decrease ($P<0.05$) of total protein and albumin. OTA doesn't affect the expression of the investigated cytokines or the other inflammatory molecules. In conclusion, 50mg/kg OTA, as the recommended guidance values by EU for OTA in pigs, administered to weanling piglets for 33 days induced some alterations of the serum biochemical parameters, with little or no effect on inflammation.

Key words: Ochratoxin A, weanling piglets, inflammation

INTRODUCTION

The Balkan endemic nephropathy (BEN) is an irreversible, chronic, tubulo-interstitial nephropathy described so far in several rural regions from the Balkan Peninsula (Bulgaria and the former Yugoslavia – Serbia, Croatia and Bosnia) and in Romania, where it affects mainly the population from Mehedinti County around the town of Drobeta Turnu Severin. As a chronical interstitial disease, BEN is characterized by a high incidence of urothelial urinary tract tumours [1]. There are scientific evidences showing that BEN is a disease induced by the environmental conditions, but the environmental factors responsible for the onset of the disease are yet to be detected; ochratoxin A is considered to be one of the factors involved in BEN triggering [2].

Ochratoxins are secondary metabolites produced by fungus. For example, in tropical and warmer regions OTA is produced by fungus of the genera Aspergillus and in temperate and colder areas by Penicillium verrucosum, [3, 4]. The most commonly occurring and most toxic member is ochratoxin A (OTA) [5]. The contamination with OTA of cereal grains (barley, wheat etc.), of the bread and spices is a real danger worldwide.

Scientific evidences showed that OTA is nephrotoxic but displays also a multiple toxicity, being genotoxic [6] or immunotoxic [7].

Among the toxins produced by Aspergillus, ochratoxins are as important as aflatoxins, in terms of toxicity being classified by IARC (International Agency for

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Research on Cancer) in the same group 2B or carcinogenic substances for humans. The reports of the European Commission (CE) show that a concentration of 14 ng/kg body weight/day is responsible for nephrotoxicity in humans (CE Regulation 1881/2006). The reabsorption of this mycotoxin takes place in the proximal and distal renal tubules. The covalent bond of OTA to the DNA is a key event for carcinogenesis induction with the formation of adducts in many tissues, which are observed in the kidney after 16 days. The kidney is the target organ of this toxin [7], and the major characteristic of BEN is the formation of multiple and bilateral tumours on the upper urinary tract [9].

Through their gut physiology very similar to human, pig represents a good model for humans. The aim of the present paper was to investigate the nephrotoxic effect of a low dose of OTA (50ppb) administrated to weanling pig.

MATERIAL AND METHODS

Twelve weanling piglets were fed a corn-soybean meal basal diet for 33 days and randomly assigned to either a control (diet without mycotoxin) or OTA (50 ppb) group. The nephrotoxic effects of OTA on the expression of some molecules involved in *i)* inflammation - like cytokines (TNF alpha, IL-1 beta, IL-8, IL-4) or other molecules involved in inflammatory processes: nuclear factor kappa-light-chain-enhancer of activated B cells - Nf-kB and inducible nitric oxide synthase- iNOS, cyclooxygenase-2 - COX2, p38 mitogen-activated protein kinases - p38, in the kidney of weaned pigs were evaluated by real-time PCR. For this purpose, kidney tissue samples were stored at −80°C until RNA extraction. Tissue samples were homogenized in liquid nitrogen using an Ultra-Turrax homogenizer and 100mg of tissue was resuspended in 1 mL TriReagent. Total RNA was extracted. cDNA were synthesized using 1µg of purified RNA and the evaluation of the effects of OTA on genes expression of above mentioned markers was realised through real-time PCR assay.

The gene-specific primer pairs were obtained from Eurogentec (San Diego, USA) [10, 11]. The relative quantification of gene expression changes was recorded after normalizing for beta 2 microglobulin (b 2 microglobulin) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression computed by using the 2(-ΔΔ CT) method and data were expressed as relative fold increase or decrease from control weanling piglets.

Total protein content was measured using Bradford assay. Purified fractions of anti-swine cytokines from: R&D Systems (Minneapolis, USA): IL-1beta (MAB6811), TNF-alpha (MAB6902), IL-10 (MAB6931), IL-4 (AF654), IL-6 (AF686), IL-8 (MAB5351) or Invitrogen (Camarillo, Canada): IFNgamma (ASC4934) were used as capture antibody in conjunction with biotinylated anti-swine cytokines: IL-1 beta (BAF 681), TNF-alpha (BAF 690), IL-10 (BAF 693), IL-4 (BAF654), IL-6 (BAF686), IL-8 (BAF 535), IFN gamma (ASC4839). Streptavidin-HRP (Biosource, Camarillo, USA) and TMB (tetramethylbenzidine) were used for detection. Absorbance was read at 450 nm using a microplate reader (SUNRISE TECAN, Austria). Recombinant swine IL-1beta, TNF-alpha, IL-6, IL-8, IFN-gamma, IL-10, IL-4 were used as standards and results were expressed as picograms of cytokine/mL, after normalization to the total protein content of the samples.

RESULTS AND DISCUSSIONS

The contamination of cereals with OTA is a worldwide problem both in tropical or temperate regions [12]. Streit et al (2013) showed that in Europe, OTA contaminated feed and raw materials in a range between 1-760 µg/kg. According to the same authors, in Romania, from 86 samples of cereals (maize, wheat, barley, oat, soya, sunflower, colza, rice, triticale, rye) tested, 18% were contaminated with OTA in a range of 81µg/kg [13]. Pigs are very sensitive to the effect of mycotoxins, and considering the OTA toxic effects, European Commission has established some guidance values for OTA concerning complementary and complete feeding stuff for pigs (50µg/kg) and for cereals and cereal products (250µg/kg).

In our trial, we wanted to investigate if the recommended guidance values by EU for OTA in pigs, 50µg/kg is responsible for nephrotoxic effects and if these results can be extrapolated to humans.

Biochemical markers play an important role in diagnosis of the kidney failure and creatinine, urea, uric acid and electrolytes represents markers of renal function for routine analysis [14]. As showed in the table 1, administration of 50µg/kg OTA to the weanling piglets has no effect on urea, creatinine, phosphorus or calcium concentration, but induced a significant decrease ($P<0.05$) of total protein and albumin. In a similar way, higher concentrations (114-300 µg /kg feed) of OTA determined in swine a significantly decrease of total protein and cholesterol associated with significantly higher serum levels of creatinine, urea, glucose, potassium and alkaline phosphatase [15, 16].

OTA induces renal, hepatic and intestinal toxicity, characterized by inflammation and cell death [17]. In our experiment we analysed if a low dose of OTA (50µg/kg) was able to induce kidney inflammation. For this purpose we have investigated the mycotoxin effect on the expression of four cytokines involved in inflammation: TNF alpha, IL-8, IL-1 beta and IL-4 and on the expression of other mediators of inflammation as p38, Nf-KB, iNOS and COX. As it can be observed in the figure 1, OTA doesn't affect the expression of the investigated cytokines or the other inflammatory molecules. These results are sustained by the effect of OTA on cytokine synthesis that was investigated using the ELISA method. Indeed, as can be seen from the table 1, OTA doesn't affect the synthesis of IFN-gamma, IL-4, IL-6, IL-8, TNF-alpha, IL-1 beta. However the synthesis of IL-10 was significantly increased after the intoxication of the weanling piglets with OTA. IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of cytokines such as TNF-alpha, IL-6 and IL-1 [18].

Indeed, other studies showed that in weaned piglets 181 µg OTA/kg feed diminished the protein content in the serum and increased levels of TNF-alpha and IL-10 in plasma [19].

Recent studies showed that OTA has inflammatory effects on kidneys (Sauvant et al., 2005). For example, long-term exposure of human kidney cells to 10 ng OTA concentrations leads to the up regulation of TNF-α [20]. Also, OTA induces phenomena typical for chronic interstitial nephropathy and activates ERK1/2, JNK, and p38 in proximal tubular cells [21]. However, in our study no effect of OTA on inflammation has been observed at 50mg/kg OTA.

Table 1 Effect of dietary OTA on selected blood biochemical parameters in piglets

Parameter	Control	OTA	P value
Creatinin (mg/dL)	0.49 ± 0.0	0.45 ± 0.02	0.159
Urea (mg/dL)	26.8 ± 2.4	21.1 ± 1.2	0.063
Phosphorus (mg/dL)	8.5 ± 02	9.0 ± 0.4	0.372
Calcium (mg/dL)	11.3 ± 0.1	13.2 ± 2.3	0.109
Total protein (g/dL)	6.0 ± 0.2	5.4 ± 0.1	0.022*
Albumin (g/dL)	3.6 ± 0.0	3.3 ± 0.0	0.039*

Table 2 Effect of dietary OTA on cytokine synthesis in the kidney of weanling piglets

Cytokines (pg/mL)	Control	OTA	P value
IFN gamma[(1)]	320.9 ± 35	266.5 ± 33	0.293
IL-4[(2)]	477.6 ± 16	366.0 ± 29	0.831
IL-6[(3)]	1749.9 ± 198	1058.3 ± 135	0.233
IL-8[(4)]	1569.2 ± 133	1316.3 ± 123	0.449
TNF-alpha[(5)]	1962.2 ± 145	1957.9 ± 106	0.981
IL-1 beta[(6)]	4679.3 ± 428	5616.6 ± 266	0.083
IL-10[(7)]	5321.5 ± 433	7806.2 ± 483	0.0025*

[(1)] interferon gamma, [(2)] interleukin 4, [(3)] interleukin 6, [(4)] interleukin 8, [(5)] tumor necrosis factor alpha, [(6)] interleukin 1 beta, [(7)] interleukin 10

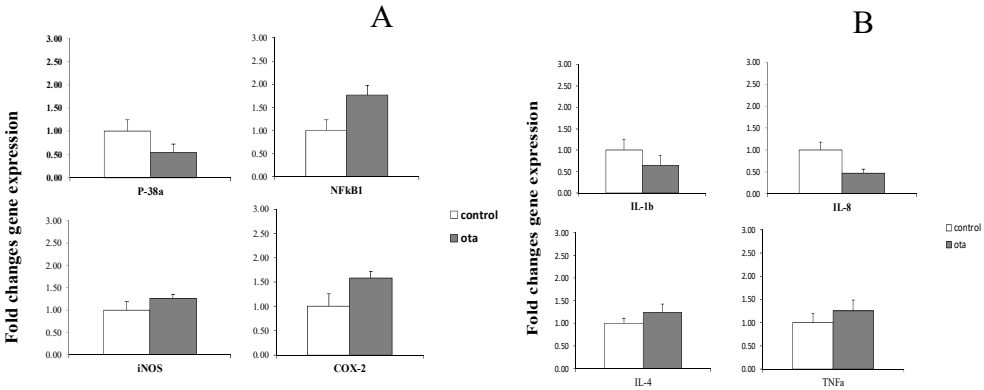

Fig. 1 Effect of OTA on markers of inflammation

CONCLUSIONS

In conclusion, 50mg/kg OTA, as the recommended guidance values by EU for OTA in pigs, administered to weanling piglets for 33 days induced some alterations of the serum biochemical parameters, with little or no effect on inflammation.

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