Effect of Ochratoxin A and Aristolochic Acid on a Porcine Kidney Cell Line

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Abstract

The Balkan endemic nephropathy (BEN) is an irreversible, chronic, tubulo-interstitial nephropathy described so far in several rural regions from the Balkan with an unknown aetiology. Both ochratoxin A (OTA) and aristolochic acid (AA) are considered responsible for BEN. This paper performed a comparison between the toxic effect of OTA and AA using a kidney cell line LLC-PK. OTA and AA decreased cell viability in a dose dependent manner. The dose dependent effect was observed for both toxins, the doses higher than 5μg/mL inducing a significant inhibition of viability ($P<0.05$) in AA and OTA treated cells, when compared with control. For doses higher than 50μg/mL, the toxicity of AA was higher than the OTA cytotoxicity. A slight decrease of IL-8 was observed after 6h, for OTA, while AA slightly increases the IL-8 synthesis. After 24h, IL-8 synthesis was decreased by both toxins, AA having a more pronounced effect than OTA. IL-10 synthesis was not affected by the treatment of both toxins. In conclusion, both OTA and AA negatively affect LLC-PK cells viability and capacity to synthesize IL-8, with AA being more toxic than OTA for the highest concentrations used in this study.

Keywords: aristolochic acid, cytokines, LLC-PK, ochratoxin A.

1. 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. With an unknown etiology, the evolution of the disease is slow and it manifests after the age of 40-50 as decompensate chronic kidney failure. The kidneys lose their capacity to eliminate the toxins from the body, which cause serious disturbances to all the organs. In most cases the disease is associated with various forms of upper urinary tract cancer. 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. It is very possible that there is a plurifactorial mechanism by which the disease occurs due to the nephrotoxic effect of the some toxins acid on the background of a particular genotype (suggested by the geographic location) and of a particular diet (high consumption of contaminated cereal grains) with improper storing facilities for the cereals and foods, which favor contamination with toxins more frequently in the rural localities. Currently, three theories try to explain the cause of this disease: (i) the hypothesis of intoxication with ochratoxin A (OTA), a mycotoxin produced by *Aspergillus ochraceus* fungi [1], which considers that BEN is induced by the intoxication of the animal and human feed and food with this toxin; the climate in southern Europe favors the growth of Aspergillus species in the detriment of Penicillium species [2]; (ii) the hypothesis of intoxication with aristolochic acid (AA), according to which the disease is produced by

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intoxication with Aristolochia (wolf's apple), a wild flower, and (iii) the hypothesis of the Pliocene lignite according to which the disease is caused by the long-term exposure to the aromatic hydrocarbons and to other toxic organic compounds from the drinking water nearby the coal mines, which the population from the endemic villages drink on a daily basis [3]. The aim of the study was the comparative study of the toxicity of both OTA and AA on a kidney porcine cell line LLC-pK.

2. Materials and methods

Cell culture and reagents. Kidney porcine cell lines (LLC-PK) from ECC were grown and differentiated Medium 199 + 2mM Glutamine + 10% Fetal Bovine Serum (FBS). Purified OTA and AA (Sigma) were dissolved in ethanol/culture media (1:1, v:v), aliquated and stored at -20^{0}C before dilution in cell culture medium.

MTT test. Cell viability in response to OTA and AA was assessed through MTT [3-(4,5-dimethylthiazol- 2-yl)- 2,5-diphenyl tetrazolim bromide] assay. Briefly, 2 × 10^5 LLC-pK cells were seeded in Medium 199 culture media in 96 well plates, incubated for 24 h at 37^{o}C and then treated with different dilutions of toxins. 24 h later, 10 μL MTT solution in PBS (5 mg/mL) was added to each well and mixed thoroughly. After incubation for 2 h at 37^{0}C, 100 μL of MTT solvent (0.1 N HCl in anhydrous isopropanol) was added to each well and plates were read within 1 h after the addition of the MTT solvent. The absorbance was measured at 570 nm using a microplate reader (TECAN SUNRISE, Austria) and the absorbance of the background at 650 nm was subtracted. All tests were performed in four independent experiments.

Neutral red test. Cytotoxicity was quantified using neutral red uptake test (NRU). 2 × 10^5 LLC-PK cells were seeded in Medium 199 culture media in 96 well plates, incubated for 24 h at 37^{o}C and then treated with different dilutions of toxins. 24 h later, the cells were fixed with 4.5% glutaraldehyde solution (SIGMA) for 30 min at room temperature and then washed with 0.9% phosphate buffered saline (PBS, SIGMA). After 30 min (room temperature) staining with 0.05% neutral red (SIGMA), acid alcohol (1% acetic acid in 50% ethanol) was added to extract neutral red from living cells. The eluted stain intensity (optical density, OD) was measured at 540 nm following at least 2 h incubation. Surviving cell number was directly related to the absorbance of the eluted stain.

Measurement of cytokine synthesis. For cytokine assessment, LLC-PK cells were seeded at 2x10^5 cells in culture media, incubated for 24h with concentration of 1μM toxins; culture supernatants were collected and frozen at -20^{o}C until analysed for cytokine content by ELISA. Purified fractions of anti-swine cytokines (R&D Systems, Minneapolis, USA): IL-8 (MAB5351), IL-10 (MAB6931) were used as capture antibody in conjunction with biotinylated anti-swine cytokines: IL-8 (BAF 535), IL-10 (BAF 693). 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-8 and IL-10 were used as standards and results were expressed as picograms of cytokine/mL. All tests were performed in four independent replicates.

One-way ANOVA tests were used to analyze the differences between control and toxins for proliferation, cytotoxicity and cytokines synthesis. The P values lower than 0.05 were considered significant. The Prism 5 software was used to calculate IC50 for both proliferation and cytotoxicity assays.

3. Results and discussion

The toxins effect on LLC-PK viability was evaluated through the measurement of the dehydrogenases enzyme activity.

As shown in the Figure 1, the addition of increasing concentrations (0-100 μg/mL) of OTA and AA decrease cell viability in a dose dependent manner.

The dose dependent effect was observed for both toxins, the doses higher than 5μg/mL inducing a significant inhibition of viability ($P<0.05$) in AA and OTA treated cells, when compared with control (Figure 1). The viability of LLC-PK cells was affected in a similar way as the viability of IPEC-1 cells, were the low dose of 1μg/mL dose, induce only a slight decrease of cell viability, while the higher doses of AA and OTA induce a significant decrease of the cell viability [4].

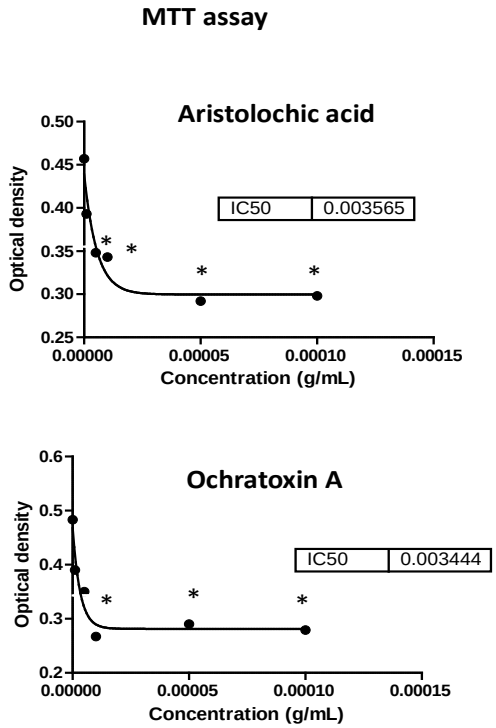

Figure 1. Effect of OTA and AA on viability

However the toxicity was higher in the IPEC-1 cells as the viability of cells treated with 100μg/mL toxin was only 35% for OTA and 30% for AA, while in LLC-PK cells, it was of 58% for OTA and 65% for AA. In conclusion, both AA and OTA induced a progressive decrease of cell viability as can be seen in the Figure 1, but the concentrations of toxins necessary to induce a 50% decrease of viability are relatively high: 3.5 mg/mL for AA and respective 3.4 mg/mL for OTA in comparison with other porcine cell lines. The low doses (1 μM) that do not induce a significant decrease of the LLC-PK viability were selected to be used in further assays as being nontoxic for the cells.

The cytotoxicity effect on LLC-PK cells was evaluated through the neutral red assay by dye neutral red. The dose dependent effect was observed for both toxins. In a similar way as for the viability test, the doses higher than 5μg/mL are cytotoxic for AA and OTA treated cells, when compared with control (Figure 2).

While the cytotoxicity of the two toxins were similar for the doses lower or equal to 10 μg/mL, for higher doses, the toxicity of AA was higher than the OTA cytotoxicity (eg. at the measuring living cells via the uptake of the vital concentration of 50 μg/mL, AA killed 76% LLC-PK cells, while OTA killed only 44% cells).

The calculation of IC 50, using Prism 5 software, shown that the concentration of toxins necessary to have 50% cytotoxicity was 4.9 mg/mL for AA and respectively 5.8 mg/mL OTA.

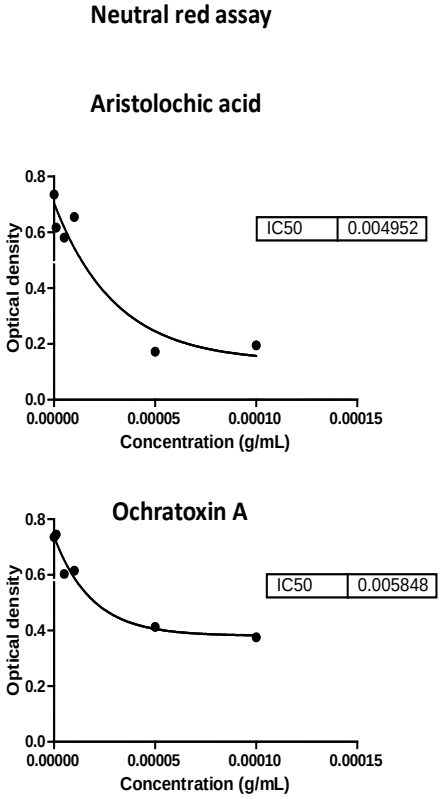

Figure 2. Cytotoxic effect of OTA and AA

Our results have been similar with those of [5] that showed a significant decrease of LLC-PK cell viability in a concentration- dependent manner after 24h of exposure to OTA. However, the same authors have shown that OTA doesn't affect the LLC-PK cell viability after 4h of incubation. Other studies [6] have shown that 2.5 μg OTA/mL significantly reduced the number of living LLC-PK1 cells to 34.5 % as compared to untreated control cells. This alteration was associated with significant changes in OTA-treated cells and release of LDH into the culture medium and apoptotic and/or necrotic cell death.

Other studies have also shown that high concentrations of AA (0.01g/L-0.08 g/L) may induce apoptosis of LLC-PK1 cells and use of calcium agonist may ameliorate AA-induced

apoptotic damage by inhibiting the increase of [Ca++] in LLC-PK1 cells [7].
Other authors [8] have shown that using the LDH release assay the highest non-cytotoxic concentration of AA was 10 μM and of OTA was 25 μM, which confirm our results.
Further we wanted to investigate the effect of the toxins on the inflammatory response through the assessment of the inflammatory cytokines synthesis. First, we have investigated the effect of the toxins on the IL-8 synthesis. IL-8 is the primary endothelial-derived chemokine involved in the recruitment of neutrophils to injured organs such as the kidneys. IL-8 is also a potent stimulant of several neutrophil cellular responses including chemotaxis, degranulation and respiratory burst [9, 10]. As can be seen in the Figure 3, IL-8 synthesis significantly increases with time. A slight decrease of IL-8 was observed after 6h, for OTA, while AA slightly increases the IL-8 synthesis. After 24h, IL-8 synthesis was decreased by both toxins, AA having a more pronounced effect than OTA.

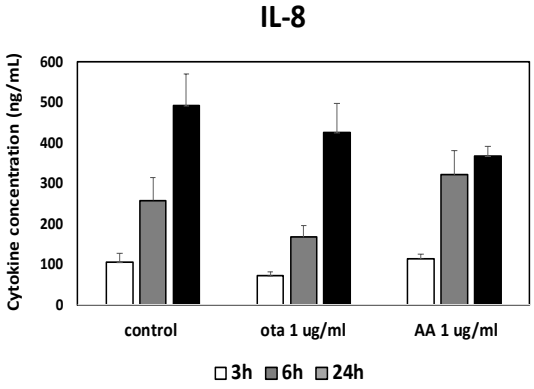

Figure 3. Effect of OTA and AA on IL-8 synthesis

Interleukin 10 (IL-10) is a cytokine with potent anti-inflammatory properties that plays a central role in limiting host immune response to pathogens, thereby preventing damage to the host and maintaining normal tissue homeostasis [11]. As can be seen in the figure 4, IL-10 synthesis increases with time. As can be seen from Figure 4, while after 3h of treatment there was and slight increase for AA and respectively a slight decrease for OTA of IL-10 synthesis, after 6 or 24h there were no effect on IL-10 synthesis. OTA induces renal, hepatic and intestinal toxicity, characterized by inflammation and cell death [12]. OTA is in principal responsible for the induction the proinflammatory cytokines as TNF alpha and IL-1 beta, while little studies have investigated the effect on IL-8 synthesis. Our results have shown that the increase of the inflammatory cytokine IL-8 was observed rather after intermediate exposure of 6h, and especially for AA, when longer exposure (24h) induces a decrease of IL-8 synthesis as comparing with the control. The increase of the inflammatory cytokines synthesis induced by nephrotoxic toxins as OTA and AA was sometimes correlated with a corresponding decrease of the synthesis of IL-10 cytokine. IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of cytokines such as TNF-α, IL-6 and IL-1 [13]. For example, Odhav et al. [14] showed a significant decrease in IL-10 synthesis in the supernatants from the neutrophils isolated from both healthy human or esophageal and breast cancer patients.

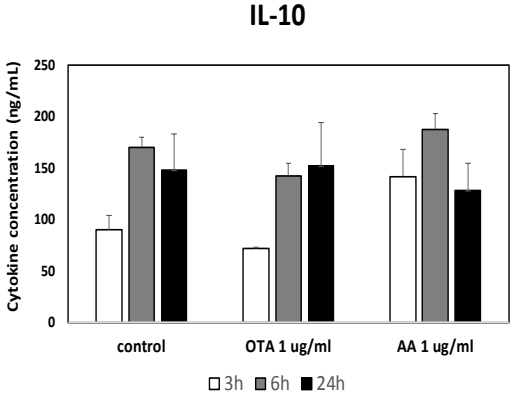

Figure 4. Effect of OTA and AA on IL-10 synthesis

However, in our study, both OTA and AA were not able to affect the IL-10 synthesis.

4. Conclusions

In conclusion, both OTA and AA induce a dose dependent effect on LLC-PK cells viability, with AA being more toxic than OTA for the highest concentrations used in this study. Both OTA and AA induced a decrease of IL-8 synthesis after 24 h of exposure, while no effect was observed for IL-10 synthesis.

Acknowledgements

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