Study of the role of aristolochic acid in triggering the porcine nephropathy

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SUMMARY

Aristolochic acid is a toxin produced by plants of the family *Aristolochiaceae*. This paper evaluates the effects of aristolochic acid (AA), a frequent grain contaminant, as a possible agent of swine nephropathy through the assessment of several markers of kidney toxicity. Ten cross-bred TOPIG hybrid were randomly distributed in two groups (five pigs/group) and fed control or 250μg AA/kg feed for 28 days. Our results have shown that AA induced changes in the renal parenchyma, while renal lesions were not found in control group. The consumption of the diets contaminated with AA significantly increase the synthesis of TNF-α and IL-8 and slightly increase the concentration of IFN gamma and IL-1 beta, while no effect was observed on IL-6 and IL-8 synthesis. Also, a significant increase of the TNF-αgene expression was observed in the kidney of AA intoxicated animals. In conclusion, our results have shown that the intoxication of weaned piglets with 250 μg AA/kg feed for 1-month results in important histopathological modifications at the kidney level and an increase in inflammatory status which can lead to the renal failure.

Keywords: aristolochic acid, swine, inflammation, nephropathy

INTRODUCTION

Porcine nephropathy is a renal pathology responsible for important economic losses in the livestock sector. The disease was identified in the 1970s in the Scandinavian countries, and has a frequency ranging from 0.6-65.9 cases/10000 pigs (Krogh, 1976). Recent cases of porcine nephropathy with a similar symptomatology and a much higher frequency have also been described in the Balkan region (Stoev, 2008). Most cases have been identified in small farms, females being more frequently affected than

males (Krogh, 1976). The disease is clinically characterized by polyuria and weight loss. Renal histology shows interstitial hypoplastic fibrosis associated with tubular atrophy and global sclerosis of glomeruli that falls from the external cortex to the internal cortex (Cosyns, 2003).

Porcine nephropathy caused by ingestion of substances was most often related to the intake of mycotoxins, secondary fungal metabolites and mainly ochratoxin A (OTA) produced by *Aspergillus ochraceus*. However, it seems that swine nephropathy has a more complicated pathology and etiology. Thus, it has been noticed that spontaneous nephropathy may be caused by some mycotoxins such as OTA, penicillin acid and fumonisin B1, which have synergistic interactions but also other non-fungal contaminants such as acid aristolohic (Cosyns, 2003). Porcine nephropathy induced by contaminated cereal ingestion has been found to be similar to human Balkan endemic nephropathy (Stoev et al., 2010a; Stoev et al., 2010b). Balkan Endemic Nephropathy (NEB) is an irreversible, chronic, tubulo-interstitial nephropathy described in several rural areas of the Balkan Peninsula (Bulgaria and the former Yugoslavia).

Aristolochic acid (AA) is a toxin produced by plants of the family *Aristolochiaceae.* Some plants belonging to the *Aristolochia* species have been used in traditional Chinese medicine as anti-inflammatory agents, diuretics, and in the treatment of edema (IARC, 2002). Aristolochic acid is traditionally used in natural remedies as an antineoplastic, antiseptic, anti-inflammatory and antibacterial agent acting as an inhibitor of phospholipase A2 (Buckingham, 2001; Cosyns, 2003). Although AA toxicity has been recognized in humans, some *Aristolochia* plants containing AAs are still being constantly used in some natural products, particularly in Chinese preparations with a wide use (Wu and Wang, 2013).

Recent studies have shown that aristolochic acid is a frequent grain contaminant (Wu and Wang, 2013), as *Aristolochia clematitis* which synthesizes aristolochic acid is a weed that can grow in wheat fields in the Balkan region and its seeds can contaminate cereals (Hranjec et al., 2005). Thus, humans can be exposed to AA by eating bread made from contaminated cereal (Grollman et al., 2007). Once AA is consumed and metabolically activated, it reacts with DNA to form adducts, (Zhou et al., 2010) the mechanism of toxicity is not well understood. In Romania, swine nephropathy is an unsettled subject, although the exposure of pigs to grain contaminated in the endemic area for NEB leads to the occurrence of this pathology. Although in Romania there is a concern regarding the determination of NEB etiology, no research has been carried out on AA-induced nephrotoxicity in pigs, as there are no data on the extent of exposure of animals to AA.

On the other hand, the existence of differences in the toxicity and carcinogenicity of aristolochic acid in different species suggests a species-

specific mechanism of action. Comparative cytotoxicity studies (Huljic et al., 2008) have shown that pig cells issued from the renal cortex can be used as an in vitro model for AA toxicity study. However, there is a very small number of *in vitro* studies using porcine kidney cells and no *in vivo* study on the nephrotoxicity of aristolochic acid in pigs.

This paper evaluates the effects of aristolochic acid (AA), a frequent grain contaminant, as a possible agent of swine nephropathy through the assessment of several markers of kidney toxicity.

MATERIAL AND METHODS

Reagents

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

Animals and treatments

For this study, a total number of ten cross-bred TOPIG hybrid [(Landrace × Large White) × (Duroc × Pietrain)] pigs with an average body weight of 10.9 ± 0.77 kg were allocated to two experimental groups (5 pigs per group). Animals were cared for in accordance with the Romanian law 206/2004 and decision 28/2011 for handling and protection of the animals used for experimental purposes. The piglets were exposed to one of the two treatments: control group (C), and aristolochic acid (AA) for 28 days. The piglets were fed a maize-soybean-meal-based diet (Table 1) contaminated or not with 250 μg AA/kg feed (Sigma). Blood samples were aseptically collected on day 28, by jugular vein puncture. Plasma obtained from blood after centrifugation at 3000 rpm, 20 min was used for the assessment of biochemical parameters. Pigs had free access to feed and water during the experimental period. At the end of the experiment (day 28), animals were slaughtered by exsanguination in an EU-licenced abattoir according with the EU Council directive 2010/63/CE. After slaughtering, samples of kidney were taken on ice and stored at –80°C until analysed for cytokine concentration, activity of enzymes involved in oxidative stress, total antioxidant capacity and lipid peroxidation. The study protocol was approved by the Ethical Committee of the National Research-Development Institute for Biology and Animal Nutrition, Balotesti Romania.

Plasma biochemical parameters

Plasma obtained from blood after centrifugation at 3000 rpm, 20 min was used for the assessment of biochemical parameters. Related to kidney functionality (creatinine, urea) were determined spectrophotometrically

from plasma on a BS-130 Chemistry analyser (Bio-Medical Electronics Co., LTD, China) using ACCENT kits (Cayman, Poland).

Table 1. Composition of experimental diet (%)

Ingredients	Control	Contaminated diet
Wheat	26	26
Corn	37.59	37.59
Rice	9	9
Soybean meal	16	16
Sunflower meal	4	4
Gluten	3	3
Salt	0.2	0.2
Monocalcium phosphate	0.9	0.9
Feed grade limestone	1.8	1.8
Methionine premix	0.05	0.05
Lysine premix	0.36	0.36
Choline premix	0.1	0.1
Vitamin mineral premix[1]	1.00	1.00
Aristolochic acid (AA) μg/kg	-	250

[1]Vitamin-mineral premix / kg diet (0-18 days): 10,000 UI vit.A; 2000 vit. D; 30 UI vit. E; 2 mg vit. K; 1.96 mg vit. B_1; 3.84 mg vit. B_2; 14.85 mg pantothenic ac.; 19.2 mg nicotinic ac; 2.94 mg vit. B_6; 0.98 mg folic ac.; 0.03 mg vit.B_{12}; 0.06 biotin; 24.5 mg vit.C; 40.3 mg Mn; 100 mg Fe; 100 mg Cu; 100 mg Zn; 0.38 I; 0.23 mg Se.

Hystopathological examinations

For the histopathological examination, samples taken from the kidneys were fixed in 10% neutral formol and included in paraffin blocks after a preliminary dehydration in solution ethanol with different concentrations (700, 800, 900, 1000) and clarification in benzene baths. The cut of paraffin blocks was performed with the Leica manual rotating microtome at a thickness of 4 m. The tissue sections were affixed to slides and stained by the Mallory trichromatic method. The histological sections were examined using the Olympus Cx41 optical microscope equipped with a digital camera and QuickPhoto Micro2.2 software for histomorphometrically analysis.

Cytokine measurement

1g of frozen kidney sample for each animal were homogenized in phosphate buffer containing 1% igepal, 0.5% sodium deoxycholate, 0.1% SDS and complete (EDTA-free) protease inhibitor cocktail tablets. The homogenates were kept 30 min on ice, and then centrifuged at 10,000 g at 4°C for 10 min. The supernatants were frozen at -200C, until analysed for cytokine content by ELISA. Total protein content was measured using Bradford assay. Monoclonal anti-porcine antibody from: i) R&D Systems (Minneapolis, USA): IL-1beta (MAB6811) and TNF alpha (DuoSet DY690B

kit) were used as capture antibody in conjunction with anti-porcine cytokines - biotinylated antibodies: IL-1beta (BAF 681), and TNF alpha (DuoSet DY690B kit). Streptavidin-HRP (Invitrogen, 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-1 beta and TNF-alpha were used as standards and results were expressed as micrograms of cytokine/g tissue.

qRT-PCR measurements

Kidney tissue samples were homogenized in liquid nitrogen and total RNAs were extracted as already described (Marin et al., 2016). 1 g of purified mRNA was transcribed into first-strand cDNA using 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). qRT-PCR was run using maxima SYBR Green/ Fluorescein qPCR Master Mix 2X (Fermentas, Thermo Fischer Scientific, USA) and 0.3 μM of both forward and reverse primers (Table 2). Gene-specific primer pairs for target genes (TNF-alpha, IL-1 beta) were obtained from Eurogentec (San Diego, USA) and found in the literature (Marin et al., 2016; Marin et al., 2013; Pistol et al., 2014). Thermal cycling was carried out with a Rotor Gene-Q Pure Detection (QIAGEN, Hilden, Germany) The relative product levels using were quantified using the 2(-ΔΔ CT) method (Livak and Schmittgen, 2001). The results are expressed as fold change (Fc) after normalization of target gene expression to the average level of expression of selected reference genes (GAPDH and Beta 2 microglobulin).

Table 2. Nucleotide sequences of primers for real-time PCR

Gene	Forward primer (from 5' to 3') Reverse primer (from 5' to 3')	Amplicon length (pb)	Genbank no.	References
Sus scrofa Glyceraldehyde 3-phosphate dehydrogenase (GAP DH)	Fw: ACTCACTCTTCTACCTTTGATGCT R: TGTTGCTGTAGCCAAATTCA	100	NM_001206359.1	Du et al., 2010
Sus scrofa Beta 2 microglobulin	Fw: GTGCTCGCGCTACTCTCTC R: GTCAACTTCAATGTCGGAT	162	NM_213978	Devriendt et al., 2009
Tumour necrosis factor alpha (TNF-α)	Fw: ACTGCACTTCGAGGTTATCGG R: GGCGACGGGCTTATCTGA	118	NM_214022	Grenier et al (2012)
Interleukin 1 beta (IL-1β)	Fw: ATGCTGAAGGCTCTCCACCTC R: TTGTTGCTATCATCTCCTTGCAC	89	NM_214055	Von der Hardt et al (2004)

Statistical analysis

ANOVA tests followed by a Fisher PSLD test were used to analyse the differences for serum parameters, gene expression, cytokine synthesis and for histomorphometrically analysis. The P values lower than 0.05 were considered significant.

RESULTS AND DISCUSSION

Necrosis has been considered one of the mechanisms for aristolochic acid-induced renal injury (Pozdzik et al., 2008; Stengel, 2010). In our study, the microscopic analysis of the kidneys area of animals intoxicated with AA reveals a number of changes in the renal parenchyma (Fig. 1). Thus, on certain territories, the parenchyma changes its architecture, aspect due to the urinary tract distention.

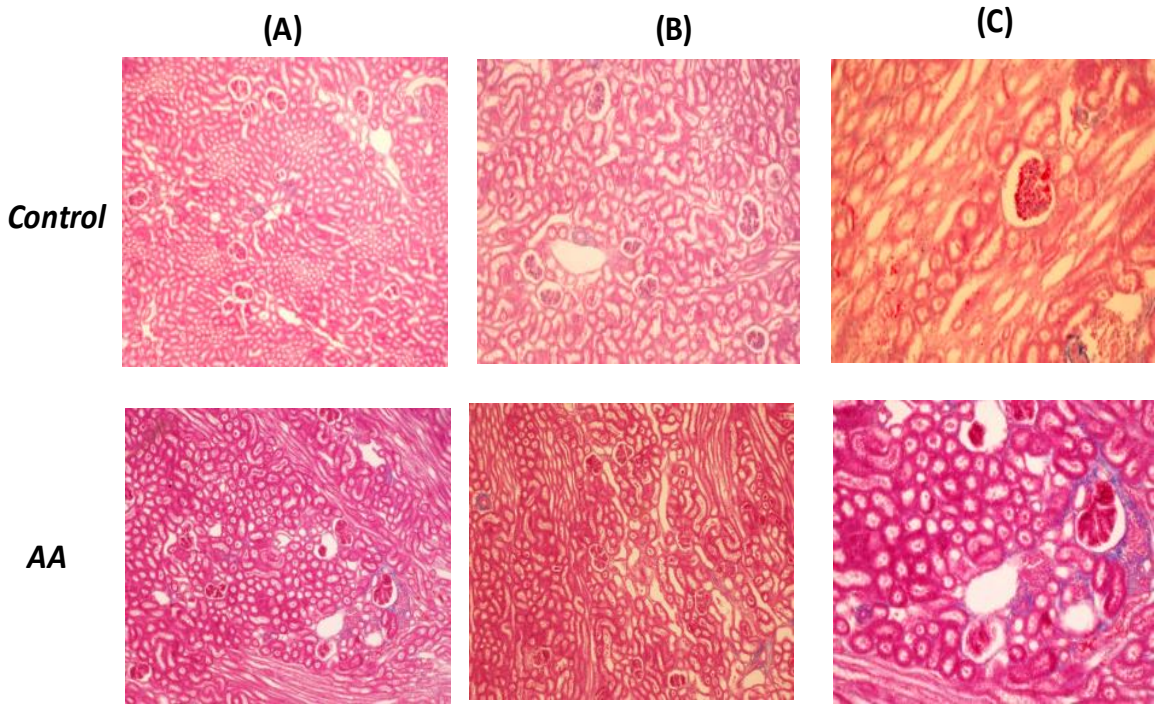

Fig. 1. *Histopathological effect of ochratoxin in the kidney of weanling piglets (Mallory trichrome stain). Renal parenchime of a control piglet (Fig 1A-control piglet). Normal and hypertrophic renal corpuscles; reducted chistic tubules (Fig. 1B-control piglet). Renal corpuscle with compressed and hemorragic glomerulus (Fig. 1C-control piglet). Heterogenic renal corpuscles, low fibrosis process, chistic tubules and mild hiperplazia (Fig 1A- AA piglet). Urine tubes with "swollen" aspect (Fig 1B- AA piglet). Heterogenic renal corpuscles, mild fibrosis processes, chistic tubules and mild hyperplasia animals, is signaled the presence of atypical cystic tubes (Fig. 1A-AA, 1B-AA and 1C-AA). The epithelial cells that trap the urinary tracts exhibit degenerative histological changes consisting of hypertrophic processes and vacuolization of cytoplasm. In the lumen of the tubes, the presence of cellular debris, with a sanguine aspect, is signaled on certain territories (Fig. 1C-AA).*

The presence of small atypical cystic tubes is signaled in the parenchyma (Fig. 1A-AA, 1B-AA and 1C-AA). The epithelial cells that trap the urinary tracts exhibit degenerative histological changes consisting of hypertrophic processes and vacuolization of cytoplasm. In the lumen of the tubes, the presence of cellular debris, with a sanguine aspect, is signaled on certain territories (Fig. 1C-AA). In some territories, kidney glomeruli are fragmented and hypertrophic, with extremely low capsular spaces, but smaller corpuscles with compressed glomeruli and large capillary spaces (Fig. 1C-AA) are also reported. A single individual of AA group exhibits interstitial hyperplasia processes (Fig. 1C-AA) on restricted territories with isolated fibrosis nuclei and vascular congestion. Renal lesions were not found in control group, renal parenchyma exhibits normal architecture, and only in small areas slight tubular hypertrophy as well as cystitis tubes of much smaller size compared to individuals of the AA group are reported.

Similar histopathological results were obtained in mice administered 3 mg AA/kg body weight every 3 days for 6 weeks (Hamouda et al., 2018). In this study, the kidney of AA treated mice showed atrophied or dilated tubules with cytoplasmic vacuolation, flattening, necrosis and even shedding and mononuclear cell infiltration in surrounding interstitial areas. This tubular injury leads to tubular atrophy, and eventually end-stage renal failure (Lin et al., 2018; Luciano and Perazella, 2015) .

Nephropathy is associated with the increase in the values of serum creatinine and urea nitrogen (Liguori et al., 2017). In our study, the ingestion of a feed contaminated with 250 μg AA /kg was not able to increase the concentration of creatinine, BUN, or to modify the ratio BUN: creatinine in the serum of intoxicated animals (data not shown) and maybe a longer exposure is necessary in order to increase these values.

Development of interstitial fibrosis that characterizes nephropathy is associated with cellular inflammation and local release of various cytokines (Eddy, 2006). In our study, the consumption of the diets contaminated with AA significantly increase the synthesis of TNF-α and IL-8 and slightly increase the concentration of IFN gamma and IL-1 beta, while no effect was observed on IL-6 and IL-8 synthesis (Table 3). Also, as can be observed in Fig. 2, a significant increase ($P<0.05$) of the TNF-αgene expression was observed in the kidney of AA intoxicated animals.

Indeed, inflammation plays an important role in the AA induced nephropathy as in AA intoxicated animals, monocytes/macrophages and T lymphocytes predominantly infiltrated areas of necrotic proximal tubular cells (Pozdzik et al., 2008). It was shown the AA causes the preferential accumulation of inflammatory cells in the interstitium of the medullary rays and of the outer medullary in renal interstitium (Pozdzik et al., 2010).

Table 3. Effect of aristolochic acid on cytokine concentration in the kidney of intoxicated animals

Cytokine concentration (pg/mL)	Control	Acid aristolochic	P value
Interferon gamma (IFN-γ)	4976 ± 358	5692 ± 189	0.0958
Interleukin 1 beta (IL-1β)	2967 ± 246	3624 ± 157	0.0741
Interleukin 6 (IL-6)	3783 ± 419	4234 ± 152	0.3919
Interleukin 8 (IL-8)	3332 ± 338	4030 ± 345	0.1873
Tumor necrosis factor alpha (TNF-α)	76.4 ± 5.2	96.4 ± 5.2	0.036

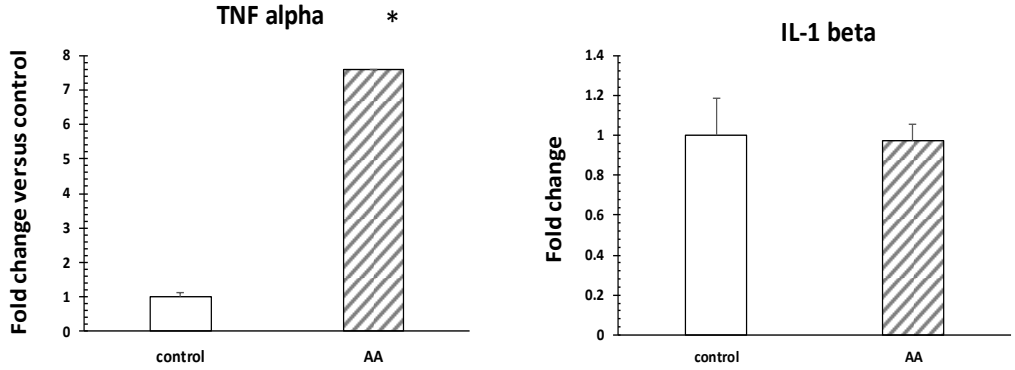

Fig. 2. *The effect of aristolochic acid on the gene expression of TNF alpha and IL-1 beta in intoxicated young piglets*

The infiltration of immune cells in the kidney interstitium was associated with in an increase of urinary excretion rates of inflammatory cytokines as IL-1α, TNFα, IFN-γ as well as IL-4 and TGF-β in AA intoxicated rats (Pozdzik et al., 2008). In conclusion, our results have shown that the intoxication of weaned piglets with 250 μg AA/kg feed for 1-month results in important histopathological modifications at the kidney level and an increase in inflammatory status which can lead to an eventually end-stage renal failure.

CONCLUSIONS

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