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Interest of metabonomic approach in environmental nephrotoxicants: Application to aristolochic acid exposure

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1. Introduction

In 1993, the occurrence of a rapidly progressive form of renal interstitial fibrosis associated with a weight loss diet which included the ingestion of pulverized plant extracts used in traditional Chinese medicine, led to the description of a new toxic nephropathy (Vanherweghem et al., 1993). The identification of aristolochic acids (AA) in these powders brought to attention the severe toxicity of certain species of Aristolochia (Vanhaelen et al., 1994). Ever since, secondary nephropathies resulting from the toxicity of plants containing AA have been described worldwide (Debelle et al., 2008). The similarity between the histological aspects of this particular nephropathy and the so-called Balkan endemic nephropathy (BEN) (Grollman et al., 2007), combined with the association between these types of renal disease and urinary tract cancers (Cosyns et al., 1999; Nortier et al., 2000), proved instrumental in reviving an old hypothesis on the aetiology of BEN. In 1969, Ivic had suggested that the latter, occurring in certain villages throughout the Danube Valley, might be caused by the chronic ingestion of the seeds of the *Aristolochia clematis,* a common plant growing in the wheat fields of these endemic regions (Ivic, 1969). This hypothesis has now been confirmed by the discovery of specific DNA adducts that are formed by the metabolites of AA in the renal tissue and the urothelial tumours of those patients suffering from BEN (Jelakovic et al., 2012).

Today, the term “aristolochic acid nephropathy” AAN is used to include any form of toxic interstitial nephropathy that is caused either by the ingestion of plants containing AA as part of traditional phytotherapies (e.g. Chinese medicine (Commission of the Ministry of Public Health, 2000; IARC, 2002), Japanese Kampo (Takako et al., 2002) and Ayurvedic medicine), or by the environmental contaminants in food (BEN) (Cosyns et al., 1994; Grollman et al., 2007). Although, initially, the Belgian cohort only included over 100 patients, it is estimated that exposure to AA affects 100,000 people in the Balkans (where the total number of patients with kidney disease amounts to approximately 25,000), 8.000.000 people in Taiwan and more than 100.000.000 in mainland China (Yang et al., 2011; Chen et al., 2012). Given the fact that the nephrotoxic effect of AA is irreversible and that their carcinogenic effects may be very slow in manifesting themselves after the patient's initial exposure, AAN and associated cancers are likely to become a major public health issue in the years to come (Gökmen et al., 2013).

Aristolochic acid nephropathy has been successfully reproduced in various experimental models. Both experimental and clinical studies indicate that the proximal tubule, particularly in its straight part (S3), is targeted by AA (Debelle et al., 2002; Lebeau et al., 2005). In 2002, Debelle et al., showed that a daily subcutaneous injection of 10 mg/kg of AA mixture (40% AAI; 60% AAII) in salt-depleted rats induced an interstitial fibrosis and renal failure within 35 days. Using the same protocol, Lebeau et al., collected urine samples to demonstrate a correlation between structural and functional injuries of the proximal tubule (increased urinary excretion rates of brush border enzymes and altered reabsorption capacity of microproteins by endocytosis). Two phases were finally observed: a first one, characterized by a transient proximal tubular necrosis (maximal at day 5), followed by an interstitial inflammation making the link with the second phase, characterized by the onset of severe tubular atrophy interstitial fibrosis (from day 7 to day 35).

Abbreviations: AA, aristolochic acid; AAI, aristolochic acid I; AAII, aristolochic acid II; AAI+AAII, mixture of aristolochic acid I and II; AAN, aristolochic acid nephropathy; BUN, blood urea nitrogen; GLUT, glucose transporter; OAT, organic anion transporter; OSOM, outer stripe of outer medulla; PTEC, proximal tubule epithelium cell; rOAT, rat organic anion transporter; SCr, serum creatinine; SGLT, sodium-glucose transporter; WRS, Wilcoxon rank-sum test.

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Although mechanistically informative, the conventional markers used in those previous studies lacked of sensitivity. When abnormal levels of those markers are noticed, it is usually too late because irreversible damaged are already reached. More recently, several authors published encouraging results showing that the metabolomic investigation of urines from animals exposed to nephrotoxicants, including AA's, was able to detect renal injuries at very early stages, also making possible some inferences in the drug-targeted biochemical pathways (Hu et al., 2017; Zhao et al., 2015a). Strategically, urine is the ideal biological fluid to discover biomarkers for renal pathologies as it is the case for AA intoxication. It can be easily and noninvasively collected in almost all patients and is well suited for kinetics studies. However, the major difficulty in using urine as an experimental matrix is the wide variability among individuals (Rucevic et al., 2012). Nevertheless, changes in the relative levels of dozens of urine metabolites may provide precious clues on cellular mechanisms responsible for damages caused to the kidneys (Wishart, 2006). This is the basis of the metabonomic approach, defined as a high speed measurement of a large quantity of low MW molecules (<1500 Da), the metabolites, present in cells, tissues and organs. The spectrometric analysis of biofluids allows the identification and quantification of dozens of metabolites in a few minutes. Discriminant metabolites identified are then related to biochemical pathways and specific biological processes.

Using this predictive omic tool, we revisited our rat models of AAs intoxication to identify early urinary biomarkers which could be further developed for a quick and a noninvasive detection of renal injuries caused by AA. The predictive aspect is of paramount importance to support patients before the onset of irreversible kidney damage.

2. Material & method

2.1. Chemicals and reagents

AAI (A5512-100 mg, Sigma-Aldrich Chemie GmbH, Germany), AAII (A3774, AppliChem, GmbH, Germany) and mixture of AAI-+AAII (40:60) (Acros Organics Co., Geel, Belgium) were dissolved with polyethyleneglycol (PEG400, Fluka Chemie, Buchs, Switzerland) in a stock solution of 20 mg/ml.

2.2. Ethics statement

All experimental protocols were approved by the Animal Ethics Committee of UMONS. Animal care and use were conducted in compliance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals. All invasive practices (subcutaneous injections, blood collections, euthanasia) were performed under isoflurane anesthesia.

2.3. Studies design & animal groups

For each study, rats (aged 4 week-old; weighting 125–150 g; supplied by Elevage Janvier (Le Genest-Saint-Isle, France)) were housed in the animal care facility within rooms kept at a controlled humidity level between 40 and 60% and a mean temperature of 21 ± 2 °C, with a 12/12-h light/dark cycle. During the quarantine week, animals had free access to standard diet (ref: 11576814, Carfil Quality, Oud-Turnhout, Belgium) and water ad libitum.

The first pilot study was designed to test several doses of the AAI and AAI+AAII. 8 Wistar Han male rats were randomly divided in three groups according to the administered substance: Group AAI (n = 3), group AAI/AAII (n = 3) and control group (n = 2) (see Table 1). Three doses were tested in this pilot study for each toxin (50, 75 or 100 mg/kg) according to a 96-h protocol (described below). In the second study, 18 Wistar Han male rats were randomly divided according to the same previous three groups: AAI 100 mg/kg (n = 6), AAI/AAII 75 mg/kg (n = 6) and control (n = 6). Compared to the pilot study, the exposure was prolonged to 192 h s (see protocol description below). Finally, in the third study, AAII toxicity was investigated following the same 96-h protocol as used in the pilot study. 12 Wistar Han male rats were randomly divided in three new groups: One control group (n = 4) and two AAII groups according to the exposed dose: 75 mg/kg (n = 4) or 100 mg/kg (n = 4). Note that the lower dose (50 mg/kg) was not been evaluated for AAII due to the lack of significant effect at this dose for both AAI and the mixture during the pilot study.

2.4. Drugs administration

After one week of acclimatization, rats were randomly allocated to the experimental groups and individually placed in metabolic cages with free access to water and 30–35 g of standard diet daily during 3 days prior to dosing. Drug administration was performed on anesthetized animals. Rats were firstly placed in an induction chamber filled with 4.0% isoflurane (lot number 37003XN, Alcyon, Villers-le-Bouillet, Belgium) at a flow rate of 1.0 l/min, then anesthesia was maintained with 1.5% isoflurane during drug administration. The AAI, AAI/AAII and AAII powders, dissolved in PEG400 (20 mg/ml), were diluted in NaCl 0,9% (50:50) before subcutaneous injection. In the control group, rats were just anesthetized without any other treatment. After dosing, rats were placed back to their metabolic cages and their body weights as well as diet and water consumptions were recorded daily throughout the study duration.

2.5. Protocols & samples collection

2.5.1. Urine samples

Urine samples were collected by fractions of 12 h-periods from the pretest days until end of study. During the collection periods, tubes were placed in refrigerated racks (4 °C) and 500 µl of a solution of 1.0% sodium azide (S8032-25 g, lot number BCBD9551V, Sigma-Aldrich Chemie GmbH, Germany) were previously added in order to avoid bacterial contamination.

In the 192-h protocol, urine samples were also collected by fractions of 12 h from pretest days up to 96 h. Then, urine collection was stopped during 48 h and resumed until the end of study.

Table 1
Summary of the experimental protocols conducted in rats.

	AAI	AAI+AAII	AAII	Control	Timing
Pilot study	50 mg/kg (n = 1) 75 mg/kg (n = 1) 100 mg/kg (n = 1)	50 mg/kg (n = 1) 75 mg/kg (n = 1) 100 mg/kg (n = 1)	X	n = 2	96 h
Second study	100 mg/kg (n = 6)	75 mg/kg (n = 6)	X	n = 6	192 h
Third study	X	X	75 mg/kg (n = 4) 100 mg/kg (n = 4)	n = 4	96 h