Toxicology

Full Paper

Serum levels of Ochratoxin A in dogs with chronic kidney disease (CKD): a retrospective study

RUNNING HEAD: OCHRATOXIN A IN DOGS WITH CKD

Meucci Valentina*, Luci Giacomo, Vanni Michele, Guidi Grazia, Perondi Francesca and Intorre Luigi

Department of Veterinary Science, University of Pisa, Italy

*Corresponding author: Valentina Meucci, Department of Veterinary Science, University of Pisa, Via Livornese lato monte, 56122, San Piero a Grado, Pisa, Italy; Tel: +39 050 2210124, Fax: +39 050 2210182; E-mail: valentinam@vet.unipi.it

ABSTRACT

Ochratoxin A (OTA) is a mycotoxin produced by secondary metabolism of several fungi belonging to the genera *Aspergillus* and *Penicillium*. OTA is potentially nephrotoxic, neurotoxic, immunotoxic and carcinogenic in several animal species and in humans. This toxin has been detected in several human food and animal feed. The aim of this study was to determine OTA in blood samples of healthy and affected by chronic kidney disease (CKD) dogs. CKD group showed higher incidence of OTA-positivity than healthy dogs (96% vs 56%) and a significantly higher median value of OTA plasma concentration (0.008 ng/m*l* *vs* 0.144 ng/m*l*). No significant correlation was observed between OTA levels and creatinine values in CKD dogs. This is first study regarding OTA detection in plasma samples of healthy and CKD dogs; the presence of this toxin is higher in nephropatic patients but is not yet clear, if it is correlated with progression of the disease.

KEY WORDS: chronic kidney disease, dog, nephrotoxin, Ochratoxin A

Chronic kidney disease (CKD) is one of the most common kidney disease in dogs, and its etiology is not fully clarified; it seem correlated with some "risk factors", such as immunomediated diseases, systemic or urinary tract infections, nephrolithiasis, drug or substance nephrotoxicity, systemic and glomerular hypertension, chronic hypoperfusion, amyloidosis and others [29]. Several well-known associations exist between CKD and both environmental agents and conditions, such as heavy metals, mycotoxins, industrial chemicals and infections. An environmental factor that may be associated with CKD in animals and human is Ochratoxin A (OTA). Ochratoxins are a group of secondary metabolites of the *Aspergillus* and *Penicillium* genera and contaminate cereals, coffee, dried fruit and other products. This group consists of OTA (Fig. 1), its methyl ester, its ethyl ester (Ochratoxin C), 4-hydroxyochratoxin A, Ochratoxin B with its methyl and ethyl esters and Ochratoxin α. OTA is the most prevalent and toxic of the ochratoxins. Initial symptoms of ochratoxicosis observed in all species include anorexia, polydipsia, polyuria and dehydration, and are associated with renal damage [32]. Upon absorption, OTAs enter the circulatory system, bind to serum proteins and accumulate in the kidneys, where they disrupt protein synthesis and other pathways in proximal tubular cells. This results in the degeneration of the proximal tubules and interstitial fibroses [32]. OTA is also known to bind with DNA molecules and induce renal tumors in animal models, although its carcinogenic mechanism remains controversial [8, 21]. IARC classified OTA as possible human carcinogen (Group 2B) [11]. Long-term exposure to OTA in humans has been implicated in balkan endemic nephropathy (BEN) and associated with urinary tract tumors, because of rather high OTA levels detected in food samples and in blood or urine from affected patients [1, 20, 28]. Pigs are the most sensitive farm animal species to the nephrotoxicity of OTA. Progressive nephropathy is seen in pigs at dietary concentrations of 1 mg/kg (equivalent to 40 μg/kg p.c.) [4]. Dog appears to be a species that is particularly vulnerable to this mycotoxin, which is well known for its nephrotoxic and immunosuppressive effects [3]. For example, a daily dose of 0.2 mg OTA/kg BW for 2 weeks or a single dose of 7.8 mg OTA/kg BW showed to be fatal to young beagle dogs [33, 34]. Clinical symptoms of the OTA poisoning included anorexia, weight

loss, vomiting, tenesmus, bloody diarrhea, increased body temperature, tonsillitis, dehydration and prostration. These findings were confirmed by another study in which dogs showed similar symptoms at OTA doses between 0.2 and 3.0 mg/kg BW [13-15]. It was reported that six dogs died in Germany in 1987, one in Scotland in 1991 and three in Korea in 2006 as a result of renal failure after consumption of feed containing OTA [9, 12, 17]. In the European Union, AFB1 is the only mycotoxin for which precise maximum limits have been established for complete and complementary feeding stuffs intended for animals [6]. With regard to the presence of other types of mycotoxins in products intended for animal feeding, including OTA, there are simple "guidance values" [7].

Several papers have been published concerning the occurrence of OTA in pet food. Razzazi *et al.* [30], quantified this mycotoxin in 60% of pet food samples (a total of 10 dry and 30 wet foods, respectively) purchased in the Polish and Austrian markets, with analogous percentages of positivity in the two types of products (40% and 43%, respectively), albeit with different levels of contamination (in the range of 0.21-13.1 μg/kg and 0.22-0.8 μg/kg, in dry and wet pet foods, respectively). Other studies conducted in Europe have shown, in contrast, a more sporadic OTA contamination in the pet food samples examined, for the most part in rather modest concentrations, always lower than 5 μg/kg [18, 22]. In a study conducted on 40 dry and wet dog foods available in the Austrian and German markets, Songsermsakul *et al.* [31] observed a range of OTA contamination (from 7 to 40 μg/kg). More recently, Gazzotti *et al.* [10] quantified OTA in 81% of 48 italian extruded pet food samples with mean concentration of 23.8 ± 9.9 μg/kg and 13.0 ± 9.7 μg/kg for standard and premium types, respectively. All the above results are below the guidance level set by European Commission, 2006.

The aim of the present study was to retrospectively assess the OTA plasmatic levels in healthy and CKD dogs.

MATERIALS AND METHODS

Case selection review: records of client-owned dogs of different breeds, sex, age and weight referred to the Mario Modenato Veterinary Teaching Hospital for nephrological consultation between December 2011 and December 2013 were reviewed. Apparently healthy dogs had been referred to the nephrology service, because of previous episodes of polyuria and polydipsia or evaluation of overall renal function before minor surgery. For each dog, data regarding history, results of biochemical analyses and urinalysis, and ultrasonographic findings were collected from the medical record to confirm the diagnosis of chronic kidney disease or to include as healthy dog. At the time of the initial examination, the dog's history was recorded and a complete clinical evaluation was performed; findings of that evaluation were included in the medical record. All dog owners were asked for informed consent so that serum and plasma samples could be stored for research purposes.

Renal panel with complete blood count (CBC) (5m*l* of blood used) and complete urinalysis performed at the time of the initial examination were obtained from the medical record of each dog; variables of interest included plasma concentrations of creatinine and urea and serum concentrations of albumin and total protein. Inclusion criteria for dogs with CKD included documented history of chronic renal disease, ultrasonographic findings and laboratory test results, indicating stable CKD for at least 3 months.

Exclusion criteria for dogs were considered a documented history of acute kidney injury (AKI), ultrasonographic findings or laboratory signs of AKI, and serum azotemia secondary to urinary obstruction or volume-responsive acute kidney injury. For dogs with CKD, the stage of disease was classified according to the 2011 IRIS guidelines on the basis of plasma creatinine concentration as follows: stage 1, < 1.4 mg/d*l* (< 123.7 μmol/*l*); stage 2, 1.4 to 2.0 mg/d*l* (123.7 to 176.8 μmol/*l*); stage 3, 2.1 to 5.0 mg/d*l* (185.6 to 442 μmol/*l*); and stage 4, > 5.0 mg/d*l* (> 442 μmol/*l*). Glomerular filtration rate was tested in each dog by means of an iohexol plasma clearance assay, and results were obtained from the medical record. In the present study, plasma iohexol clearance < 60 m*l*/min/m2 was considered to represent a decreased GFR [16, 25].

High-performance liquid chromatography method

OTA (from Aspergillus ochraceus) (M 403.8) reference standard was purchased from Sigma (Milan, Italy). The OTA standard was dissolved in a toluene-acetic acid mixture (99:1 %, v/v) to give a stock solution of 200 µg/m*l* which was stored at -20°C until use. Working solutions were prepared by diluting daily the stock solution with the mobile phase consisting of methanol-sodium phosphate buffer (pH 7.5) 50:50 % v/v. HPLC-grade water, methanol and acetonitrile were purchased from VWR (Milan, Italy). The chromatographic system consisted of Jasco 880 pump and Jasco 821 fluorescence detector (Jasco, Tokyo, Japan). JascoBorwin software was used for data processing. The excitation wavelength (λex) and emission wavelength (λem) were set at 380 and 420 nm. The reversed-phase column was a Luna C18 ODS2, 3 µm, (4.6 x 150mm) (Phenomenex® Torrance, CA, USA). The column was kept at room temperature. The HPLC was operated with mobile phase system consisting of methanol-phosphate buffer solution pH 7.5 (0.03 M Na2HPO4, 0.007M NaH2PO4) 50/50 % v/v at flow rate of 1 ml/min. The HPLC method was validated according to [2]: selectivity, linearity, limits of determination (LOD) and quantification (LOQ), repeatability and reproducibility were determined. Calibration curves were based on the analysis of triplicate standards solution at 7 concentration levels in matrix. Plasma samples spiked with OTA at 0.025, 0.05, 0.1, 0.25, 0.5, 1 and 2 ng/ml were analyzed using extraction and HPLC method. The experiment was repeated 5 times. Taking into account concentration step, plasma spiked samples corresponded to OTA standard concentrations of 0.1, 0.2, 0.4, 1, 2, 4 and 8 ng/m*l*. The repeatability was tested by analyzing samples of plasma spiked with OTA at the levels of 0.05 ng/m*l* (corresponding to 0.2 ng/m*l*), 0.25 ng/m*l* (corresponding to 1 ng/m*l*) and 2 ng/m*l* (corresponding to 8 ng/m*l*). All samples were measured in triplicates on the same day. For the within-laboratory reproducibility test, each of the contamination level was tested in triplicates in seven days. The results of these experiments were used also for the determination of the recovery. Selectivity studies have been expressed as the ability to assess unequivocally OTA in the presence of components which may be expected to be present: it has been evaluated by the comparison of free-OTA vs

spiked samples.

Samples preparation

OTA was extracted according to [24]. One ml of plasma was mixed with 5 ml of MgCl2 (0.1 M)-HCl (0.05 M) pH 1.5 solution and 5 m*l* of ethylacetate, vortexed for 1 min, shaked for 10 min on horizontal shaker, and then centrifuged for 10 min at 3,000 rpm. The organic phase was removed, the residue was re-extracted, with 5 m*l* of ethylacetate, and the organic phases were combined. Ethylacetate was evaporated to dryness under nitrogen stream and reconstituted in 250 µ*l* of mobile phase and a 100 µ*l* aliquote was injected into HPLC system.

OTA confirmation

For OTA positive samples, confirmation was done by OTA methyl ester formation. One hundred µ*l* of mobile phase reconstituted sample were mixed with 300 µ*l* of BF3 solution and the mixture was heated at 70 °C for 20 min; 50 µ*l* of the mixture was then assayed for OTA-Me. Confirmation was based on the disappearance of the OTA peak, and the appearance of a peak corresponding to the OTA-Me.

Statistical analysis

Statistical analysis was performed with a standard software program. All data were tested for normality by means of the Kolmogorov- Smirnov test. The data are reported as mean and standard deviation. A value of $p < 0.05$ was considered significant. Variance analysis among mean values of OTA in healthy dogs and in dogs with IRIS stage 1, 2, 3 or 4 disease was performed by means of a ANOVA test followed by a Dunn multiple comparison test. Linear regression analysis and Spearman correlation coefficient analysis were used to assess the correlation of plasma creatinine and urea concentration and OTA levels for dogs at each stage of the IRIS classification.

157

158 **RESULTS**

159 *High-performance liquid chromatography method*

160 The calibration curve was established in the range of 0.025 to 2 ng/m*l* (r = 0.995). Limit of
161 detection was equivalent to 0.0125 ng/m*l*. Average recovery was 81.9 ± 3.2%, 89.7 ± 0.8% and
162 82.3 ± 1.5% respectively, in plasma samples enriched with OTA at the levels of 0.05, 0.25 and 2
163 ng/m*l* (Table 1). All results were corrected for recovery. Each positive sample was confirmed by the
164 OTA methyl ester formation, that showed disappearance of the OTA peak (retention time: 8.5 min)
165 (Fig. 2) and appearance of the OA methyl ester peak (retention time: 14 min).

166 *Case classification*

167 Of 102 dogs referred for nephrological consultation between December 2010 and December
168 2013, 46 were included in the study. 18 dogs of these had definitive diagnosis of CKD on the basis
169 of azotemia, low urine specific gravity, renal proteinuria (urinary protein-creatinine concentration
170 ratio, > 0.5) and abnormal renal morphology at ultrasonographic examination.

171 All 18 dogs were classificated and divided in different stages of kidney disease (according to
172 IRIS guidelines) as follows: stage 2, 5 dogs; stage 3, 6 dogs; and stage 4, 7 dogs.

173 In other 28 dogs, no abnormalities were detected by CBC, biochemical analyses, urinalysis
174 or ultrasonography, although some dogs had history of previous episodes of polyuria and
175 polydipsia. However, for purposes of the present study, plasma iohexol clearance < 60 m*l*/min/m2
176 was GFR assessments and 5 of these 28 apparently healthy dogs were considered to have early
177 CKD and were classified as IRIS stage 1 (plasma creatinine concentration < 1.4 mg/d*l* [123.7
178 μmol/*l*]). Twenty-three dogs were considered healthy and used as controls.

179 All dogs with stage 2, 3 or 4 disease received specific treatment for chronic kidney disease
180 (antacids, antiemetics, vitamin B complex and renal diet) according to the severity of clinical signs.
181 The most common clinical signs were polyuria, polydipsia, weakness, dysorexia and vomiting.
182 Polyuria, polydipsia and weakness were present in all dogs with stage 2 CKD and only 1 dog had

183 gastrointestinal signs.

184 *OTA concentrations*

185 Data regarding plasma OTA concentration were tested for normality (Kolmogorov-Smirnov
186 test) and did not follow a Gaussian distribution. Rates of OTA-positive samples, median values and
187 ranges of OTA plasma concentrations are given in Table 2. The CKD group exhibited both a
188 significantly ($p < 0.01$) higher incidence of OTA-positivity than healthy subjects (96% vs 56%) and
189 a significantly ($p < 0.001$) higher median values of plasma concentrations (0.008 ng/m*l* *vs* 0.144
190 mg/m*l*). The highest OTA plasma concentration is observed in a CKD dog (1.05 ng/m*l*, i.e. 130-
191 fold the median value in healthy group).

192 Rates of OTA-positive samples, median values and ranges of OTA plasma concentrations
193 for healthy dogs and dogs with stage 1, 2, 3 and 4 are given in Table 2. The median OTA for
194 healthy dogs and dogs with stage 1, 2, 3 and 4 disease differed significantly (Kruskal-Wallis test; p
195 $= 0.0002$). Results of a Dunn post test indicated that there was a significant ($p < 0.01$) difference in
196 OTA levels between healthy dogs and dogs with IRIS stage 1 disease and between healthy dogs and
197 dogs with stage 4 disease, but not between healthy dogs and dogs with stage 2 or 3 disease dogs (p
198 > 0.05) (Fig. 3).

199 Spearman tests revealed no significant correlation between plasma OTA and both creatinine
200 ($p = 0.20$) and urea ($p = 0.11$) plasma concentrations in healthy and CKD dogs (Fig. 4).

201

DISCUSSION

Mycotoxins and related pathologies have become a worldwide issue and raise serious sanitary problems. Studies from several countries have attempted to investigate human exposure to OTA. Two approaches were undertaken: the analysis of food and the measurement of OTA in biological fluids. The later was used to evaluate association of human exposure to OTA and existence of human diseases, especially nephropathy [5, 19, 26]. The significance of OTA levels in human plasma as a marker of OTA intake can however be questioned. Although Szczech *et al.* [32, 33] and Jeong *et al.* [12] have highlighted how the OTA exposure in dogs can result in serious kidney damage, to the author's knowledge, this is the first study regarding OTA determination in plasma of dogs with different stages of CKD. In the present study, CKD patients showed both higher rates of OTA positivity and median values than the healthy population. Özçelik *et al.* [26] analyzed human plasma samples obtained from patients with different diseases of the urinary tract (CKD, bladder tumors and urinary) and from healthy controls. A statistically significant difference between the values of OTA present in the healthy and the sick ones was observed, with particular reference to CKD patients. The values of OTA detected in the blood of sick human patients, as well as those of healthy patients, are comparable with the values that we found in our study in the respective groups of dogs. The present results indicate the potentially contamination of food for animals with this toxin. OTA is a contaminant found not only in vegetable foods, but also in matrices of animal origin, as a result of the accumulation of these compounds in muscles, organs and offal (kidneys and liver, in particular), which are often used in high quantities by the pet food industry, especially for the formulation of wet products [23, 27]. The results obtained in this study suggest that OTA can have a role in the onset of kidney disease in dogs and the statistically significant difference between patients with CKD and healthy can be attributed to decreased glomerular filtration which increases the half-life of this toxin and could exacerbates its own toxicity.

In our study, OTA plasma concentration didn't correlate with serum creatinine and urea. Furthermore, a statistically significant difference between healthy and dogs at stage 1 and stage 4 of kidney disease was observed, while no difference was observed between healthy and dogs with stages 2 and 3. These results may be explained, because of the high variability in median OTA concentration in groups 2 and 3 and also because of the small number of patients in each group.

Biomonitoring is usually used to assess internal OTA exposure resulting from dietary intake and from other sources. Mycotoxin levels in blood and/or urine provide good estimates of past and recent exposure, since OTA binds to serum proteins and is also partly excreted via the kidney. But, measuring OTA alone does not reflect its biotransformation. The metabolism of OTA in animals and humans remains to be further investigated. Metabolism of OTA in rodents, especially in rats, was extensively investigated. However, the information in other species is not enough, especially for companion animals as dogs. Currently, no report on metabolism of OTA in dogs is available. Further studies should be carried out to understand its metabolism in companion animals.

Our results show that OTA is detected in almost all plasma samples tested and suggest that OTA contamination is widespread in foods consumed by these animals. For these reasons, regular controls should be enforced and exposure to OTA should be kept to a minimum, avoiding the consumption of heavily contaminated foods. From an epidemiological point of view, OTA plasma levels are considered a short-term biomarker with a high within-subject variability; therefore, they have limited use at the individual level. However, OTA measurements can be used to characterize populations or subgroups of subjects, particularly in prospective studies storing plasma samples taken at baseline, well before possibly related outcomes occur. Additional studies of dietary determinants of OTA intake in animals are needed and the correlation between OTA levels and the consumption of specific food should be explored.

Conflict of Interest: The authors declare that they have no conflict of interest.

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353 Figure legend:

354 Fig. 1. Chemical structure of OTA.

355 Fig. 2. Chromatogram of a OTA naturally-contaminated serum sample of a CKD dog (line A) and
356 OTA standard dilution (10 ng/ml in HPLC mobile phase) (line B).

357 Fig. 3. Concentrations of OTA in canine plasma of healthy dogs and IRIS stages 1, 2, 3 and 4 CKD
358 dogs; Different letters (a, b) are used to report statistically significant differences ($p < 0.05$).

359 Fig. 4. Spearman correlation analysis between plasma creatinine (mg/dl) and urea (mg/dl) and
360 serum OTA levels (ng/ml) in healthy and CKD dogs.

361

362 **Table 1. Validation parameters for HPLC method of OTA in canine plasma samples.**

Parameters	
LOD	0.0125 ng/m*l*
LOQ	0.0250 ng/m*l*
r^2	0.995 (n = 5 replicates)
CV % (intra-day)	
0.05	5.8 (n = 3 replicates)
0.25	8.9 (n = 3 replicates)
2.00	4.3 (n = 3 replicates)
CV % (inter-day)	
0.05	9.7 (n = 21 replicates)
0.25	8.3 (n = 21 replicates)
2.00	7.0 (n = 21 replicates)
Recovery %	
0.05	81.9 ± 3.2 (n = 21 replicates)
0.25	89.7 ± 0.8 (n = 21 replicates)
2.00	82.3 ± 1.5 (n = 21 replicates)

363 LOD: limit of detection; LOQ: limit of quantification; CV: coefficient of variation.

364

365 **Table 2. Concentrations of OTA in plasma of healthy, CKD and IRIS stages 1, 2, 3 and 4**

366 **dogs.**

Group	N.	Median (range) ng/m*l*	% of positives
Healthy	23	0.008 (<LOD-0.27)	56% (13 of 23)
CKD	23	0.144 (<LOD-1.05)	96% (22 of 23)
IRIS stage 1	5	0.580 (0.007-1.050)	100% (5 of 5)
IRIS stage 2	5	0.050 (<LOD-0.686)	80% (4 of 5)
IRIS stage 3	6	0.101 (0.031-0.683)	100% (6 of 6)
IRIS stage 4	7	0.221 (0.061-0.725)	100% (7 of 7)

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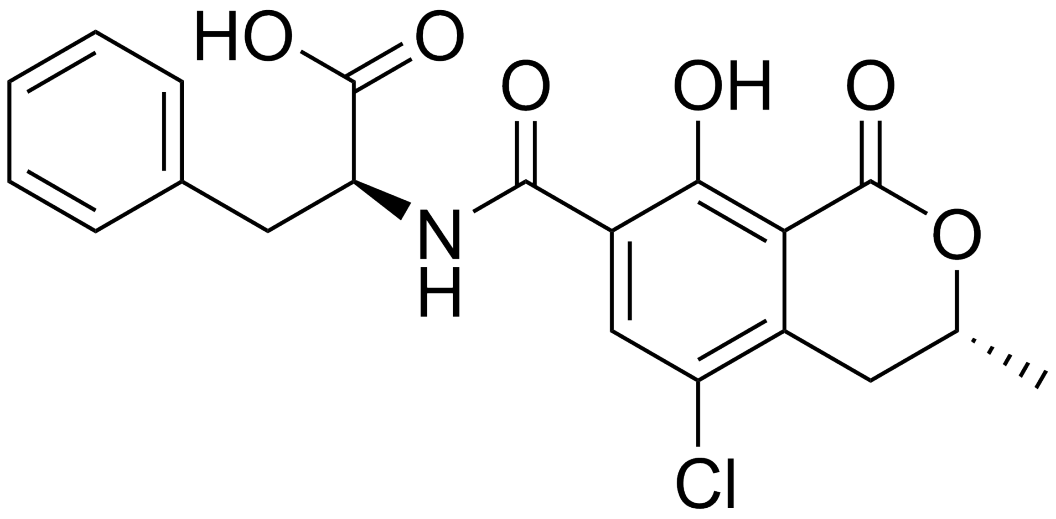
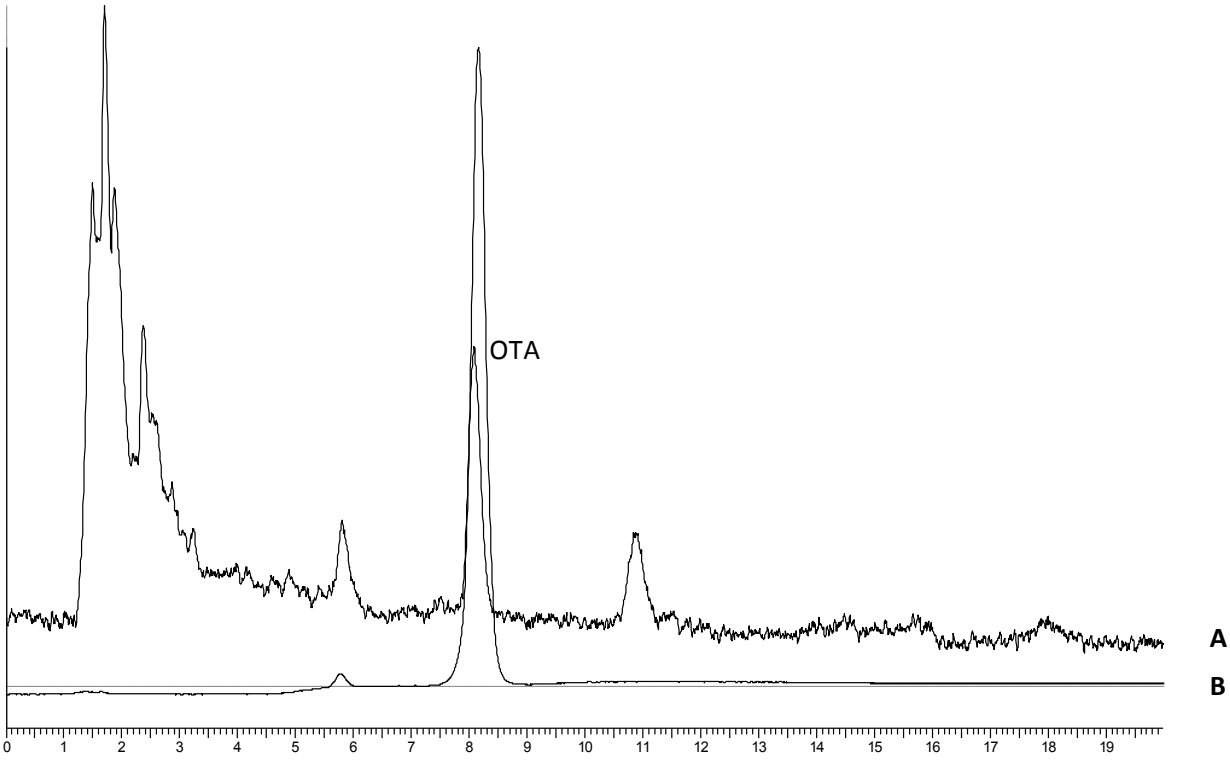


Fig. 1



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375 **Fig. 2**

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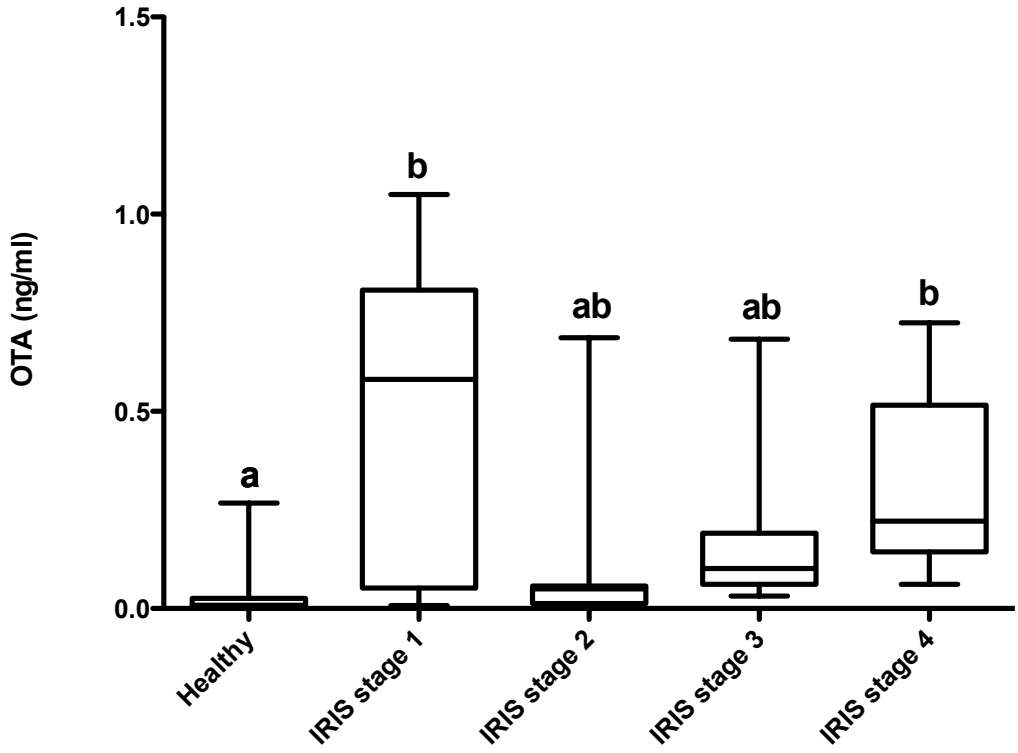

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379 **Fig. 3**

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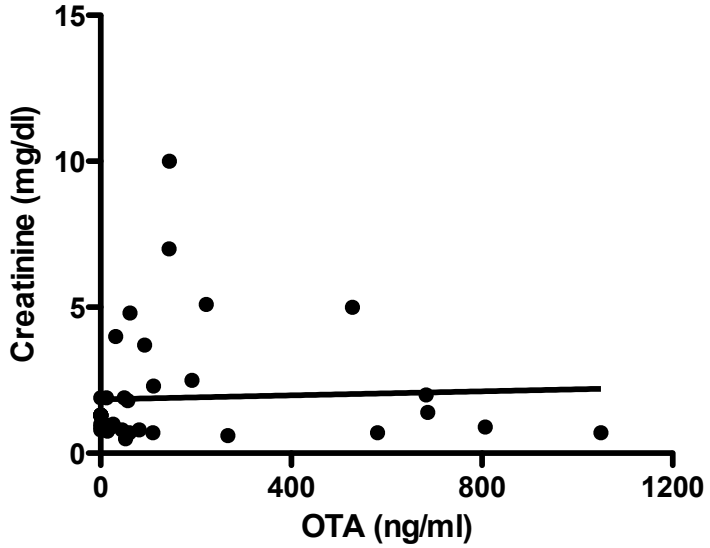

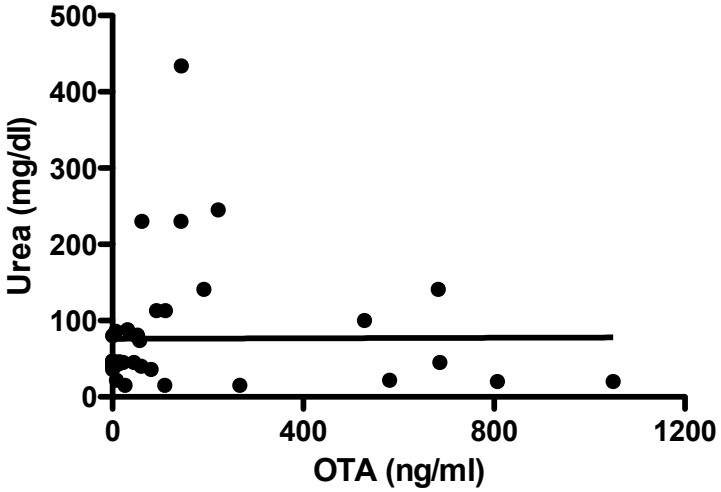

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383 **Fig. 4**

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