Food and Chemical Toxicology 111 (2018) 363–373

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Sex differences in ochratoxin a toxicity in F344 rats after 7 and 21 days of daily oral administration

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ARTICLE INFO

Keywords:
Ochratoxin a (OTA)
Sex differences
Hormones
Kidney transporters
Gene expression
Mycotoxins

ABSTRACT

Ochratoxin A (OTA) is a potent renal carcinogen in male rats but not in females. The mechanisms underlying these differences are unknown. The sex-dependent response of F344 rats after a repeated OTA oral administration for 7 (0.50 mg/kg bw) or 21 days (0.21 and 0.50 mg/kg bw) was evaluated. General toxicity, sex and thyroid hormones and histopathology were studied. OTA was quantified (HPLC-FLD) in plasma, kidney and liver and the expression of kidney transporters (RT-qPCR) was studied. After 7 days, kidney histopathology showed more pronounced signs of toxicity in males than in females. After 21 days, a higher toxicity was observed but sex differences disappeared. OTA concentration in plasma and tissues was similar in both sexes. Downregulation was the general OTA-induced effect. Oats' downregulation was slow in males and Oat3 did not change in females. Oatp1 was strongly downregulated in males after 21 days. An opposite effect was observed in Bcrp after 21 days: downregulation in males and upregulation in females. Females showed a dose- and time-dependent decrease of progesterone. Despite the sex differences, the final balance in OTA toxicokinetics at renal cell level does not seem to support a higher accumulation of OTA in male kidneys.

1. Introduction

Ochratoxin A (OTA) is a well-known mycotoxin produced by species of *Aspergillus* and *Penicillium* genera, mainly *A. ochraceus* and *P. verrucosum*. It can contaminate a great variety of vegetal products, especially grains, and enter the food chain through several commodities. OTA is considered nephrotoxic, hepatotoxic, immunosuppressive, neurotoxic, and teratogenic (EFSA, 2006). However, the main concern regarding OTA toxicity is its carcinogenic potential. The International Agency for Research on Cancer classified OTA as a possible human carcinogen (group 2B) (IARC, 1993), the National Toxicology Program 14th Report on Carcinogens as reasonably anticipated to be a human carcinogen (NTP, 2016), and Lock and Hard (2004) regarded this xenobiotic as one of the most potent renal carcinogens in rodents. However, significant sex and species differences in kidney sensitivity have been described: male rodents are more sensitive than females and rats are considerably more sensitive than mice (EFSA, 2006). Unfortunately, relatively little information is available to allow an understanding of the mechanisms that underlay these differences, and also, few short-term toxicity studies have been carried out in rats of both sexes.

Based on the organ- and site-specificity of OTA effect, ochratoxin uptake by kidney transporters has been proposed as a key event in the mode of action of the mycotoxin. Indeed, this event has been suggested to explain some of the sex- and species-differences in sensitivity towards OTA (EFSA, 2006; Mally, 2012).

Several studies have demonstrated that OTA can be transported by Slc22 (Oat), Abc, Slco1 (Oatp) and Slc15 (Pept) protein families (for a review, Anzai et al., 2010), and interestingly, most of them show species-, organ- and/or sex-dependent expression. Sabolic et al. (2007) reviewed gender differences in kidney function, especially, expression of transporters in rats. In general, despite some variation between studies, Oat1, Oat3, Mrp2, Mrp4, Bcrp and Oatp1 show higher expression in males, while Oat2, Oat5 and Pept2 are more expressed in female rats. On the other hand, some toxicogenomic studies have shown a clear downregulation of some transporters, mainly Oat1, Oatp1 and Mrp2, after OTA exposure in male rats treated for 7 or 21

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https://doi.org/10.1016/j.fct.2017.11.003
Received 15 June 2017; Received in revised form 26 October 2017; Accepted 3 November 2017
Available online 07 November 2017

days with 0.30 or 0.50 mg/kg bw (body weight) (Arbillaga et al., 2008; Marin-Kuan et al., 2006). Moreover, a dual effect has been observed in Oat transporters expression depending on the dose in male Wistar rats treated every 2nd day for 10 days: upregulation at low doses (25, 50 μg/kg bw) and downregulation at high dose (500 μg/kg bw) (Zlender et al., 2009). In addition, after a single administration of 0.50 mg/kg bw, a sex- and time-dependent profile was observed in some transporters. The most prominent change affected Oat2 expression that was upregulated in males and downregulated in females after 48 h (Pastor et al., 2016).

Several studies have demonstrated that hormones affect transporters expression, which might explain some sex differences previously described. The expression of Oat1, Oat3 and Oatp1 was increased by testosterone and inhibited by estrogens (Kudo et al., 2002; Ljubojevic et al., 2004; Lu et al., 1996). In contrast, estrogens enhanced Oat2 and Oat5 expression, whilst testosterone decreased their expression (Breljak et al., 2010; Kudo et al., 2002; Ljubojevic et al., 2007). On the other hand, progesterone increased Oat1, Oat2 and Oat5 expression, did not affect Oat3 and diminished Bcrp and Pept1 (Breljak et al., 2010; Ljubojevic et al., 2004, 2007; Watanabe et al., 2006; Wu et al., 2013). Besides sex hormones, Pept1 and Pept2 expression was shown to be decreased due to thyroid regulation (tri-iodothyronine, T_3, and thyroxine, T_4) (Lu and Klaassen, 2006). On the other hand, some studies have demonstrated that OTA could interfere with hormone production (Frizzell et al., 2013; Gharbi et al., 1993; Hassan et al., 2010; Kumar et al., 2011; Mor et al., 2014; Woo et al., 2013). However, OTA-induced effect is uncertain because different models (*in vitro* or *in vivo*), doses or time of exposure have been used.

Thus, to gain further insight into the molecular mechanisms of the early stage of OTA renal toxicity, the aim of the present study was to evaluate the sex-dependent response of F344 rats after a repeated OTA oral administration for 7 (0.50 mg/kg bw) or 21 days (0.21 and 0.50 mg/kg bw). For that purpose, general toxicity, urine and biochemistry parameters and histopathology of both target (kidney) and non-target (liver) organs were evaluated. OTA was quantified in plasma, kidney and liver samples by high-performance liquid chromatography - fluorescence detection (HPLC-FLD), in order to correlate its levels with the lesions found. Moreover, the expression of the main transporters was studied in kidneys, by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR), for a better understanding of their role in mycotoxin accumulation after repeated administration, as well as their possible expression change due to both OTA and/or hormone regulation. And finally, sex and thyroid hormones have been quantified for evaluating their possible impact in the final outcomes regarding histopathological lesions and transporters regulation.

2. Materials and methods

2.1. Chemicals

Ochratoxin A was obtained in powder from Sigma-Aldrich (Steinheim, Germany). It was dissolved in sodium bicarbonate ($NaHCO_3$ 0.1 M pH 7.4) (Sigma-Aldrich Steinheim, Germany) for animal treatment and in methanol HPLC grade (Sigma-Aldrich Steinheim, Germany) for HPLC analysis stock solutions. Then, it was aliquoted and maintained at −20 °C until use.

All reagents used for the HPLC analysis were of pro-analysis grade. Absolute ethanol, trichloroacetic acid 20%, sodium acetate anhydrous (CH_3COONa), ortho-phosphoric acid (85%) and sodium hydroxide (NaOH) were purchased from Panreac (Barcelona, Spain). Acetonitrile and methanol HPLC grade were obtained from Sigma-Aldrich (Steinheim, Germany). Sodium dihydrogen phosphate monohydrate (NaH_2PO_4*$1H_2O$) was obtained from Merck (Darmstadt, Germany). Normal saline solution (NaCl 0.9%) was obtained from Braun Medical SA (Barcelona, Spain). Type I water (Ultramatic Type I, Wasserlab Navarra, Spain) was used to prepare all aqueous solutions. The aqueous phases were filtered through a 0.45 μm nylon membrane filter (Teknokroma, Barcelona, Spain). Samples were filtered before injection into HPLC with low protein binding Durapore® (polyvinylidene difluoride, PVDF) 0.45 μm membrane filter from Millex®-HV (Merk Millipore Cork, Ireland).

2.2. Safety precautions

Due to its toxicity, OTA has always been handled in solution, thereby avoiding the formation of dust and aerosols. Nitrile gloves and FFP3 masks were used for all procedures carried out and during the manipulation of treated animals or contaminated samples.

2.3. Animals

This study was approved by the Ethics Committee on Animal Experimentation of the University of Navarra. All animals were supplied by Charles Rivers Laboratories (France). Eleven-week-old male and female Fisher 344 (F344/IcoCrl) rats of 260.93 ± 12.00 and 157.98 ± 8.52 g, respectively on day of arrival (table 1 supplementary data), were used for the repeated oral dose study. At the commencement of the study the weight variation of the animals did not exceed ± 20% of the mean weight of each sex, as recommended by OECD guidelines (OECD, 2008). Females were not synchronized for estrous cycle. Moreover, twelve-week-old male F344 rats (275.27 ± 7.48 g on day of arrival) were used for obtaining biological samples for HPLC-FLD method revalidation. All animals were used after a week of acclimatization, housed in polycarbonate cages with stainless steel covers, under standard conditions (temperature 22 ± 3 °C, humidity 50 ± 20%, 12 h light/dark cycle) and provided with sterile food (Harlan) and water *ad libitum*.

2.4. Treatment and study design

Animals (n = 60) were randomly distributed into ten groups of 6 animals (5 groups of males and 5 groups of females). Following a week of acclimatization, animals were daily gavaged (maximum volume of 1 mL/0.1 kg bw) with their corresponding treatment for 7 or 21 days. In one hand, two groups of each sex were treated for 7 days respectively with the vehicle ($NaHCO_3$ 0.1 M pH 7.4) (control group), or with 0.50 mg of OTA/kg bw (high dose group). On the other hand, three groups per sex were administered for 21 days: control, low dose (0.21 mg of OTA/kg bw) and high dose (0.50 mg of OTA/kg bw). Rats were daily weighted for adjusted administration while food and water consumption were measured once a week. For urine collection, rats were transferred into metabolic cages 12 h prior to necropsy without food but with free access to drinking water. After 24 h of the last administration, animals were euthanized by decapitation. Unfortunately, two rats should be euthanized for ethical reasons while the study was carried out. For that reason, at the end of the 21 days study, male control and female low dose groups had 5 animals instead of 6.

Urine volume was measured and an aliquot was immediately used for biochemical analysis. The remaining urine was aliquoted and stored at −40 °C for future studies.

Blood from decapitation was collected into heparinized tubes (BD Vacutainer system, K3E 5.4 mg Plus Blood Collection Tubes Plymouth, UK) and centrifuged (1085 × *g* for 15 min at 4 °C) in order to obtain plasma for biochemical analysis and OTA quantification. For hormones quantification, blood was collected into serum separator gel tubes (Venosafe™, Terumo® La Rioja, Spain), allowed to clot for 20 min and centrifuged (695 × *g* for 10 min at room temperature) for serum obtention. Both, plasma and serum samples, were stored at −20 °C until analysis.

Heart, spleen, liver, adrenal glands, kidneys, testes or ovaries and thymus were removed and weighted. Brain and lungs were removed

and flash frozen in liquid nitrogen. For this study, right kidneys were cleaned with water, until external blood was removed, and then cut longitudinally. One half of right kidney, a piece of liver, and all other organs were fixed in formaldehyde 3.7–4.0% w/v buffered to pH 7 and stabilized with methanol (Panreac Barcelona, Spain) for histological evaluation. The other kidney half and a piece of liver, were also cleaned with water, weighted and flash frozen in liquid nitrogen for HPLC-FLD OTA quantification. The reasons for only cleaning with water externally and not flushing by syringe the pieces of organs intended for HPLC-FLD analysis were: a) to mimic the conditions of the toxicity studies as recommended in the ICH guideline S3A (ICH, 1994) and b) to reproduce previous studies of our group (Vettorazzi et al., 2008, 2011) and others (Li et al., 1997; Han et al., 2013). Left kidney and the remaining parts of liver were cut into small pieces and flash frozen in liquid nitrogen for nucleic acids extractions. All frozen tissues were stored at −80 °C. All the dissection material was cleaned with water and rinsed with ethanol after each animal necropsy in order to prevent contamination between samples.

2.5. Urine and plasma biochemistry, hormone determination and histopathology

Fresh urine samples were analyzed using urine test strips (Combur Test® M cobas®, Mannheim, Germany) in Cobas U411 analyzer (Roche Diagnostics) for semi-quantitative determination of specific gravity, pH, leucocytes, erythrocytes, nitrite, protein, glucose, ketone bodies, urobilinogen, bilirubin and blood.

Plasma biochemical analysis was performed with a Hitachi 911™ (Roche Diagnostics) analyzer using standard protocols according to the manufacturer's instructions. Urea, creatinine, total protein, albumin, bilirrubin, glucose, cholesterol, aspartate transaminase (AST) and alanine transaminase (ALT) were determined.

Sex [progesterone (ng/mL), estradiol (pg/mL) and testosterone (ng/mL)] hormone concentration was measured by a solid-phase competitive chemiluminescent enzyme immunoassay (Immulite 2000; Siemens Healthcare Diagnostics Inc.). The method has a relevant specificity for progesterone with a cross reactivity for androstenedione, corticosterone, 17α-hydroxy-progesterone and testosterone < 0.45%. Moreover, estradiol and testosterone assays presented an imprecision within run < 10% and < 16.3% in each case, and a cross reactivity for many hormones and derivates < 1.9% for estradiol and < 2.0% for testosterone.

On the other hand, thyroid hormones, T_3 and T_4 (nmol/L), were measured by an electrochemiluminescence immunoassay "ECLIA" with Cobas 601 immunoassay analyzer. The assay employs a competitive test principle with polyclonal antibodies specifically directed against the hormone. Endogenous hormone (T_3 or T_4) competes with the added biotinylated T_3-or T_4-derivative for the binding sites on the antibodies labeled with the ruthenium complex. Imprecision of the assay was < 3.1% for T_3 and < 1.8% for T_4 and analytical specificity for other thyroid hormones and derivates was < 0.16% and < 1.4% in T_3 and T_4 respectively.

For the histopathological analysis, kidney and liver sections fixed in 10% neutral buffered formalin were dehydrated and embedded in paraffin. Paraffin sections (3 μm) were cut, mounted onto glass slides, and dewaxed and stained with hematoxylin and eosin (H&E) for the subsequent examination. Histopathological examination was carried out by an animal pathologist (J.A García Jalón) following the terminology recommendations of the European College of Veterinary Pathologists.

2.6. HPLC-FLD method revalidation and OTA quantification in plasma, kidney and liver samples

For OTA quantification a previous specifically validated method (Vettorazzi et al., 2008) for rat biological samples was revalidated according to the following parameters: linearity, precision and accuracy (within- and between-day variability), selectivity and recovery in plasma, kidney and liver samples. The procedure employed for evaluating these validation parameters was the same as in the validation process and it is summed-up below. A pool of blank samples obtained from eight male F344 rats (13-week-old not treated) was used for preparing calibration samples for recovery experiments.

OTA standard solution (100 mg/L) was obtained after dissolving 5 mg of OTA in 50 mL of methanol. The concentration was tested spectrophotometrically at 330 nm (MW = 403.8 ε = 5500 M^{-1} cm^{-1}). Different working standard solutions were prepared by dilution in methanol of this standard solution.

Two calibration curves of six points each (ranges of 2–20 and 20–200 μg/L) were performed using three replicates of calibrations standards for each point. They were prepared by evaporating 200 μL of the corresponding working standard solutions with a centrifugal vacuum concentrator (miVac Duo Concentrator, GeneVac Ipswich, Suffolk, UK) for 15 min at 60 °C. Then, they were dissolved in 200 μL of a solution 1:5:8 (trichloroacetic 20% - normal saline solution - absolute ethanol), similar to supernatants obtained after OTA extraction from real samples. Linearity was verified by: correlation coefficient (r^2 > 0.999), relative standard deviation (RSD) between response factor (< 5%), slope interval (p = 95%) not having to include zero. Moreover, three replicate calibration standards at 2, 6, 20, 60 and 200 μg/L were analyzed on 1 day (within-day) and on 3 different days (between-day) to study precision and accuracy of the linearity. The data was acceptable when accuracy was within ± 10% SE (standard error of the mean) and precision (RSD%) was ≤10%.

Selectivity was verified by comparing the chromatograms obtained from blank samples, blank samples spiked with OTA and OTA-treated rat samples.

For determining the recovery of the method, plasma, kidney and liver samples were fortified with known OTA concentrations: 8.4, 56 and 560 μg/L in plasma and 56, 224 and 2240 μg/L in kidney and liver. For that purpose, an adequate volume of the corresponding working standard solution were evaporated for 15 min at 60 °C using a centrifugal vacuum concentrator and dissolved into 250 μL of blank plasma, kidney or liver tissue homogenate (prepared as described in next paragraph) by mixing, first for 2 min in vortex and then for 15 min in a rotating shaker. Afterwards, OTA was extracted and analyzed. The recovery value (%) was calculated by dividing measured concentration by theoretical OTA concentration. OTA could be detected, under quantification limit, in blank plasma pool, so this response was subtracted from spiked samples areas. Moreover, the repeatability and the reproducibility of the method were studied. Three spiked samples of each concentration were measured on 1 day and on 3 different days for each matrix. Recovery values should be within 80–120%.

Tissue samples preparation was made using thawed pieces of kidney and liver (200–400 mg) homogenized for 1 min in a round-bottom plastic tube with 4 μL sodium phosphate buffer (0.05 M, pH 6.50, adjusted with NaOH) per mg of tissue in a T25 Ultra-turrax Digital High Speed Homogenizer (IKA®, Germany). Then, homogenates were aliquoted and stored for at least 1 day at −80 °C until OTA extraction. For that purpose, frozen plasma or tissues homogenates were kept at room temperature for 30 min. Then, 400 μL of ice-cold absolute ethanol and 50 μL of 20% trichloroacetic acid were added to 250 μL of sample in order to allow protein precipitation and OTA release. Then, they were mixed for 2 min using vortex and for 15 min in a rotating shaker, and centrifuged (3900 × g for 15 min at 4 °C) for protein precipitation. Supernatants were filtered with low protein binding Durapore® (PVDF) 0.45 μm membrane filter from Millex®-HV (Merk Millipore) and injected into the HPLC-FLD system. With this extraction, plasma and tissues homogenates were diluted 2.8. The final dilution factor for kidney and liver samples was 11.2 taking into account the homogenization process and the extraction volumes used. Moreover, as high OTA concentration was expected in plasma samples from the *in vivo*

study, an additional dilution (1:60) was made with a solution 1:5:8 (20% trichloroacetic acid - normal saline solution - absolute ethanol). In this case, the final dilution factor for plasma was 168.

HPLC analyses were performed on an Agilent Technologies 1100 liquid chromatographic system equipped with a fluorescence detector (model G1321A) controlled by Chemstation 3D software. OTA was analyzed on a 5 μm (25 × 0.4 cm) Tracer Extrasil ODS2 column with a Tracer Extrasil ODS-2 precolumn, both from Teknokroma (Barcelona, Spain). The mobile phase was 29:29:42 (v/v) methanol - acetonitrile - sodium acetate (5 mM acidified to pH 2.6 with ortho-phosphoric acid) and the flow rate was 1.5 mL/min. The injection volume was 100 μL. Chromatography was performed at 40 °C and the fluorescence detection conditions were: Ex = 225 nm and Em = 461 nm. In these conditions, OTA retention time was 4.5 min.

2.7. Kidney transporters expression quantification by RT-qPCR

Gene expression analysis were made using kidney samples obtained from 7 and 21 days control and treated (0.50 mg/kg bw/day) rats of both sexes. Transporter families Slc22 (Oat1, Oat2, Oat3, Oat5 and Oat8), Abc (Mrp2, Mrp4 and Bcrp), Slco1 (Oatp1 and Oatp2) and Slc15 (Pept1 and Pept2) were quantified using RT-qPCR as described in Pastor et al. (2016). Briefly, pieces of approximatively 50 mg of frozen kidneys were homogenized in TRIzol® (50 mg/mL of TRIzol) (Invitrogen™, USA) using a T25 Ultra-turrax Digital High Speed Homogenizer (IKA®, Germany). RNA was isolated according to the TRIzol® manufacturer's protocol, purified using the RNeasy® Mini kit (Qiagen, Hilden, Germany) and finally dissolved in RNase-free water. Samples quantity and purity was measured spectrophotometrically (SmartSpec™ Plus, Bio-Rad). All samples showed A260/A280 ratio between 1.8 and 2.0. Afterwards, they were frozen at −80 °C until DNase treatment was carried out according to DNA-*free*™'s protocol (Ambion® by Life Technologies, USA). For that purpose, 2 μg of RNA (volume: 10 μL) were treated with 1 μL of rDNase I plus 1 μL of DNase I Buffer at 37 °C for 20 min to remove any DNA contamination. Then, cDNA was synthesized following manufacturer's instructions of High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems™, USA) in StepOne™ Real-time PCR System (Applied Biosystem®, Life Technologies), used as thermocycler. The first strand cDNA synthesis was started with random primer annealing at 25 °C for 10 min. Secondly, reverse transcriptase reaction (RT) was carried out using MultiScribe™ Reverse Transcriptase for 120 min at 35 °C. Finally, the enzymes were inactivated at 85 °C for 5 min. Each reaction was accompanied by a no-RT control in which the reverse transcriptase was replaced by RNase free water. cDNA samples were stored at −80 °C.

For RT-qPCR, all plasticware consumables, TaqMan® probe set, as detection and quantification method, and TaqMan® Universal PCR Master Mix were obtained from Applied Biosystems™, USA. The final PCR reaction mix included 4.5 μL of diluted cDNA sample, 0.5 μL TaqMan® "best coverage" gene expression assays for each specific cDNA amplification (Pastor et al., 2016), plus 5 μL of TaqMan® Universal PCR Master Mix at a final volume of 10 μL. Reactions were performed with the StepOne™ Real-time PCR System following the next program: first, a holding stage of 2 min at 50 °C followed by 10 min at 95 °C for DNA denaturation and *Taq* polymerase activation, secondly, the cycling state of 40 cycles for amplification at 95 °C for 15 s and 60 °C for 1 min. Samples were then cooled to 40 °C for 30 s. Sample maximization method (as many samples as possible and one gene analyzed per run) was used as the run layout strategy. Each sample was measured in duplicated and inter-run calibrators (samples repeated in every run), no-template and no-RT controls were included.

Ct (threshold cycle) values were obtained and analyzed with StepOne Software v2.2.2 (Applied Biosystems™, Life Technologies). Actb, Gapdh, 18S, Ppia and Ubc were evaluated to select the best reference gene. Finally, Ppia was chosen as the most stable gene according to different approaches [Coefficient of variation (CV), Microsoft Excel NormFinder v0.953 (Andersen et al., 2004) and RefFinder (Xie et al., 2011)], and it was used to normalize the expression of target genes. Relative mRNA quantification of each target gene was calculated by the application of the formula $2^{-\Delta\Delta Ct}$ ($\Delta\Delta C_t$ method). First, C_t value was normalized to the reference gene (ΔC_t), and then it was quantified according to a calibrator ($\Delta\Delta C^t$), which is the sample used in each case as the basis for comparison.

2.8. Statistical analysis

Data are presented by descriptive analysis as mean ± standard deviation (SD) of animals. However, semi-quantitative urine biochemistry parameters and histopathological findings are summed up in frequency tables. Statistical analysis was performed by using software SPSS 15.0, using Kruskal Wallis *H* test followed by Mann-Whitney *U* test. A p-value < 0.05 was accepted as the level of significance.

3. Results

3.1. General toxicity study

No clinical signs of toxicity were observed during the study. In addition, OTA treatment did not affect food and water consumption (data not shown). However, high dose treated males showed a decrease of body weight gain, significant and continuous after day 14 of administration (Fig. 1).

In order to analyze possible organ weight changes due to mycotoxin administration for 21 days, absolute and relative weights of heart, spleen, liver, adrenal glands, kidneys, testes or ovaries and thymus were studied (data not shown). In terms of absolute weight, heart, liver and both kidneys were smaller than control organs in males and both kidneys and right ovary were smaller in treated females than in control ones. But taking into account relative organ weights, only a significant dose-dependent increase of testes was observed in males and a light dose-dependent significant decrease of right kidney was observed in females.

Blood biochemistry (table 2 supplementary data) and urianalysis did not show significant changes, except in the number of erythrocytes and leucocytes that were found in urine of treated rats with respect to control animals (Table 1). This effect was more evident in males than in females. Moreover, a slightly elevated proteinuria was found in treated males (Table 1).

Sex (progesterone, testosterone and estradiol) and thyroid (T_3 and T_4) hormones were measured in serum samples. As expected, testosterone in females and estradiol in males, were lower than quantification limit. Regarding progesterone levels (Fig. 2A) a significant dose- and time-dependent decrease was observed in females. In contrast, although

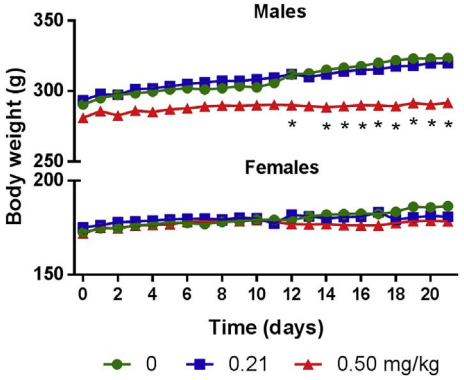

Fig. 1. Body weight (bw) evolution of F344 rats after a daily oral administration of the vehicle ($NaHCO_3$) or OTA (0.21 or 0.50 mg/kg bw) for 21 days. In each sex, treated groups were compared to their control. Differences were considered statistically significant (*) when p-value < 0.05 (Mann-Whitney *U* test).

Table 1
Presence of erythrocytes, leucocytes and protein in urine of male and female F344 rats after a daily oral treatment with vehicle ($NaHCO_3$) or OTA (0.21 or 0.50 mg/kg bw) for 7 and 21 days. The number of erythrocytes and leucocytes per μL and the protein content in mg/dL is presented (in brackets the number of animals with the same value).

Time (days)	7				21					
Sex	Males		Females		Males			Females		
Dose (mg/kg)	0	0.50	0	0.50	0	0.21	0.50	0	0.21	0.50
Animals (n)	6	6	6	5	4	6	6	6	5	6
Erythrocytes (/μL)	10 (3)		10 (1)	10 (1)	10 (3)					
	25 (2)			25 (3)	25 (1)		25 (1)		25 (3)	25 (5)
		50 (3)				50 (6)	50 (3)		50 (2)	50 (1)
		150 (3)		150 (1)			150 (2)			
Leucocytes (/μL)	25 (2)			25 (1)	25 (1)		25 (1)		25 (3)	25 (5)
	100 (3)	100 (2)		100 (2)	100 (3)	100 (4)	100 (1)		100 (1)	100 (1)
		500 (4)		500 (1)		500 (2)	500 (4)			
Protein (mg/dL)	25 (5)	25 (3)	25 (3)	25 (3)	25 (2)	25 (3)	25 (1)	25 (2)	25 (3)	25 (3)
	75 (1)	75 (3)		150 (1)	75 (2)	75 (3)	75 (4)			

less pronounced, some 21-day treated males showed a dose-dependent tendency to increase progesterone level (Fig. 2A). No significant changes were observed in testosterone levels in males (Fig. 2B) or of estradiol in females (Fig. 2C). Finally, our results did not show any changes in T_3 and T_4 levels after OTA treatment (data not shown).

Liver and kidney tissues of all the animals were analyzed. In liver, a very mild increase in the number of isolated necrotic cells was observed in treated animals with respect to the controls, and this fact was slightly more frequent in males than in females. Also, diffuse inflammatory infiltrates were localized both in parenquima and in portal space with a higher frequency in animals treated for 21 days than in control and 7-day treated animals, but these differences might be not relevant. As can be seen in Table 2, the most important findings were concentrated in kidneys. In 7-day treated males, a mild or very mild mesangial hyperplasia (Fig. 3A:a) was detected. This effect was observed in 5/6 males and in none of the females (Fig. 3A:b). After 21 days treatment, glomerulonephritis was observed in 6/6 males (Fig. 3B:a) and 5/5 females treated with 0.21 mg/kg bw, and in 3/6 males and 1/6 females treated with 0.50 mg/kg bw. Regarding, collecting ducts injury, mild or moderate alterations were observed in almost all the treated animals, with slight sexual differences after 7 days of treatment (Fig. 3A:e and f) but without a clear dose-response effect (Table 2). Nevertheless, the most significant findings were found in proximal and distal tubules. In all the 7-day treated males and females, proximal tubule cells presented degeneration and necrosis (Fig. 3A: c and d) (Table 2). After 21 days, in addition to proximal tubulonephrosis, intense alterations of the distal tubules were evident: lining cell degeneration could be observed with necrotic and sloughed cells detached in the tubule lumen and the presence of karyomegalic cells (Fig. 3B: c, d, e and f). In particular, animals treated with the low dose, showed clear signs of distal tubule cells proliferation (Fig. 3B: a and b).

3.2. HPLC-FLD method revalidation and OTA quantification in biological samples

The method was selective in plasma, liver and kidney samples and no interference peaks appeared in blank matrices. All the requirements for linearity were verified for the two calibration curves generated. Precision and accuracy of the linearity also presented adequate values (less than 10%) both within- and between-day (Table 3 supplementary

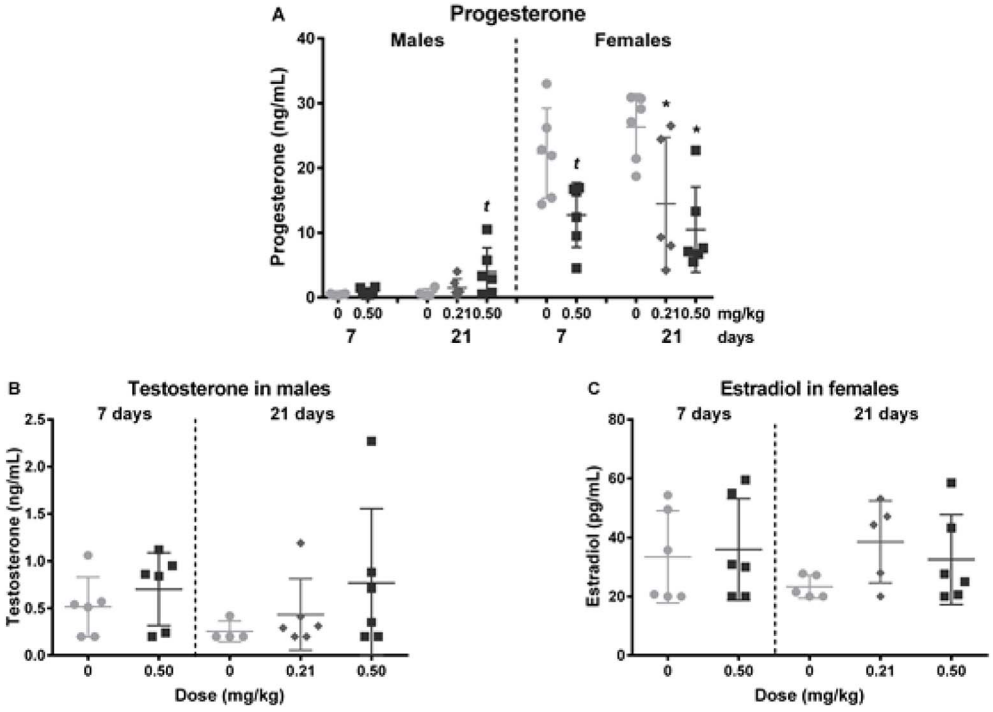

Fig. 2. Hormone quantification in F344 rats after a daily oral administration of vehicle ($NaHCO_3$) or OTA for 7 (0.50 mg/kg bw) or 21 days (0.21 or 0.50 mg/kg bw). A) Individual progesterone levels (ng/mL) in males and females. B) Individual testosterone levels (ng/mL) in males. C) Estradiol levels (pg/mL) in females. In each sex, treated group was compared to its control. Differences were considered statistically significant (*) when p-value < 0.05 or nearly to be significant (t) when p-value $= 0.055$ (Mann-Whitney U test).

Table 2
Histopathological findings in kidney samples obtained from male and female F344 rats after a daily oral treatment of OTA for 7 (0.50 kg mg/kg bw) or 21 days (0.21 or 0.50 mg/kg bw). The lesions found were evaluated based on the following grading system: unobserved or imperceptible (−) rare or very little intense or very mild (+) infrequent, less intense, discrete, mild (++) moderately frequent or moderate (+++) fairly frequent or severe (++++) very frequent or very intense (+++++). Normal histology was observed in control groups kidneys. *ID: animal identification.*

Treatment	ID	Males			Females		
		Glomerulo-nephritis	Tubulo-nephrosis	Collecting duct injury	Glomerulo-nephritis	Tubulo-nephrosis	Collecting duct injury
7 days	*1*	–	++	+++	–	++ [a]	–
0.50 mg/kg	*2*	++	++	++	–	+++++	–
	3	++	++	++	–	+++	+
	4	+	++++	+	–	+	+
	5	+	++++	++	–	+	++
	6	++	++++	+	–	++	+
21 days	*1*	+	+++	+	+	++++	+
0.21 mg/kg	*2*	+	++++	+	+	++++	+
	3	+	++++	+	+	++++	+
	4	+	+++++	+	+	++++	+
	5	+	++++	+			
	6	+	++++	+	+	++++	+
21 days	*1*	–	+++	+	–	++ [a]	–
0.50 mg/kg	*2*	–	++++	+	–	++++	+
	3	–	++++	+	–	++++	+
	4	+	+++++	+	–	++++	+
	5	+	++++	+	++	++++	+
	6	++	++++	+	–	+++	+

[a] Steatosis.

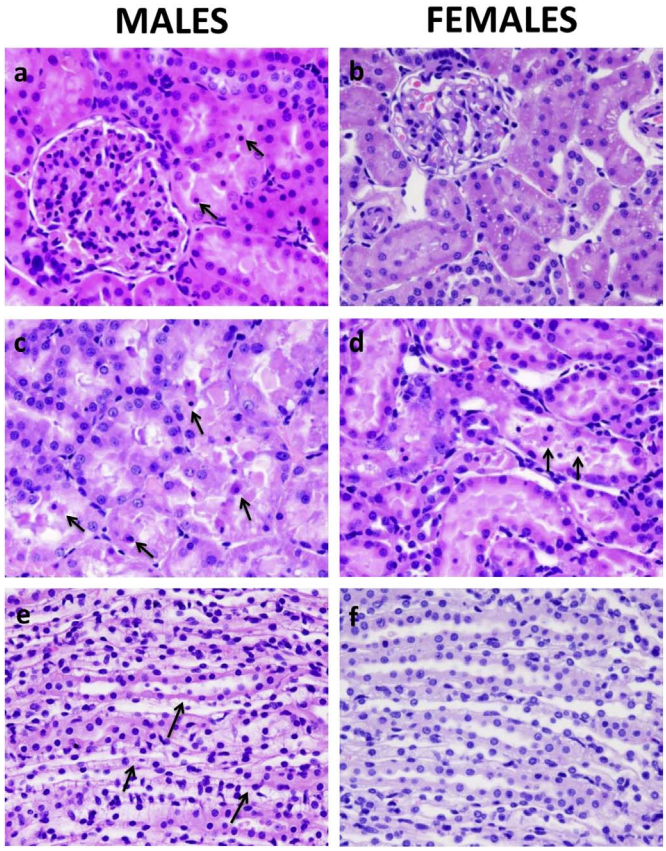

Fig. 3A. Main degenerative kidney lesions in animals treated with OTA (0.5 mg/kg bw) for 7 days. a. Mild mesangial hyperplasia of glomeruli and mild degeneration of proximal tubules (arrows) (H. E. X 400, male 2). b. Normal glomeruli with mild degeneration of proximal tubules (H. E. X 400, female 1). c. Severe degeneration and single cell necrosis of proximal tubules (arrows) (H. E. X 400, male 4). d. Moderate degeneration of proximal tubules with necrotic cells in the lumen (arrows) (H. E. X 400, female 3). e. Mild vacuolation in collecting ducts (arrows) (H. E. X 400, male 2). f. Normal collecting ducts (H. E. X 400, female 1).

data). Moreover, recovery study was made for each matrix (Table 4 supplementary data), and was very efficient (94.14% for plasma, 96.79% for kidney and 95.59% for liver) and precise (RSD obtained in within- and between-day experiments were below 10%) in all the matrices studied.

This analytical procedure was applied to measure plasma, kidney and liver samples of the present study and results are presented in Fig. 4. OTA distribution pattern was the same in all the matrices studied. Very high concentrations were measured in plasma, and lower levels were found in livers and kidneys. As expected, a higher time of exposure of the same dose and a higher dose with the same time of exposure produced significant higher OTA levels in plasma, liver and kidney. Thus, the increase of OTA concentration in all the matrices was dose- and time-dependent. Regarding sex differences, they are not evident. Comparing both sexes, only after 7 days a statistically significant higher plasma and liver concentrations could be detected in females compared to males (Fig. 4).

3.3. Gene expression analysis by RT-qPCR

3.3.1. Kidney transporters expression at basal level

Basal transporters expression was analyzed in all the control animals of this study, in order to confirm or discard sex or age related differences under our experimental conditions. To this purpose, gene expression of kidney transporters in male and female control animals was studied and compared. The results are presented in Fig. 5. An illustration of the relative expression of each transporter, together with its membrane localization and transport direction is shown in Fig. 7.

No statistically significant differences were observed in mRNA levels of 15-week-old animals (21 days) compared to 13-week-old ones (7 days).

With respect to sex differences, marked significant differences were observed for some transporters at both timepoints. Regarding Slc22 family transporters, Oat2 was much more expressed in females than in males. To a lower extent, Oat5 was also more expressed in females than in males. In the Abc family, Bcrp expression was significantly lower in females than in males. And in the Slco1 family, Oatp1 was not expressed in females.

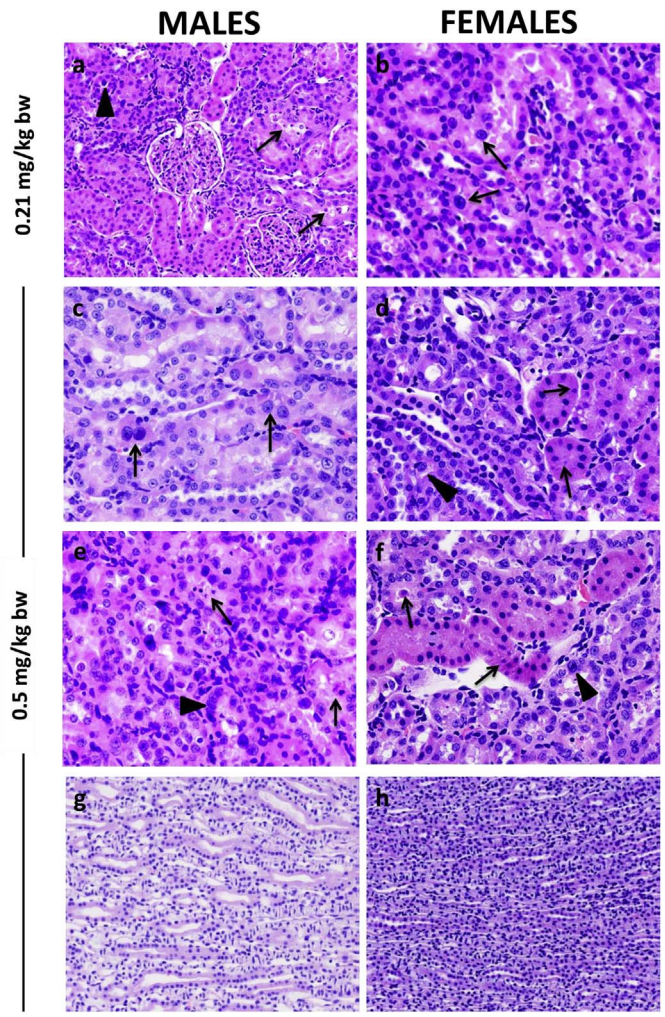

Fig. 3B. Main degenerative kidney lesions in animals treated with OTA (0.21 or 0.5 mg/kg bw) for 21 days. a. Very mild mesangial hyperplasia of glomeruli, with severe degeneration of proximal tubules (arrow); regeneration and karyomegalic cells in distal tubules (arrowhead) (H. E. X 200, male 2). **b.** Regeneration and karyomegalic cells (arrow) in distal tubules (H. E. X 400, female 1). **c.** Distal tubules with karyomegalic cells (arrows) (H. E. X 400, male 1). **d.** Proximal tubules with single cell necrosis (arrows) and distal tubules with karyomegalic cells (arrowhead) (H. E. X 400, female 1). **e.** Proximal and distal tubules degeneration, necrosis (arrows), cell proliferation (arrowhead) and karyomegalic cells (H. E. X 400, male 2). **f.** Proximal tubules with single cell necrosis (arrows) and distal tubules with karyomegalic cells (arrowhead) (H. E. X 400, female 2). **g.** Very mild collecting ducts degeneration. (H. E. X 200, male 1). **h.** Normal collecting ducts (H. E. X 200, female 1).

3.3.2. Kidney transporters expression profiles after 7- and 21-day OTA treatment

Kidney transporters gene expression was studied in male and female F344 rats administered with a daily oral dose (0.50 mg/kg bw) for 7 or 21 days. In order to see if OTA treatment induced any change in the expression of these genes, and to determine if this change was sex related, and how it was modulated by treatment time, different comparisons were performed. Results are presented in Figs. 6 and 7.

The most important changes affected Slc22 and Abc families. OTA produced a clear general downregulation of Oat transporters in both sexes, with the exception of Oat3, that did not show any difference in the mRNA levels in females. Also, comparing both sexes responses after 7 and 21 days, Oats downregulation was slower in males than in females. Regarding Abc family, after 21 days, Mpr2 expression increased in both sexes, although this increment started before in males than in females, and Bcrp expression showed opposite response in both sexes, decreasing in males and increasing in females. Another important difference between both sexes is the significant downregulation of Oatp1 transporter only in males after 21 days of treatment.

4. Discussion

The objective of this study was to unravel some molecular mechanisms that may underlay the different response to OTA toxic insult that has been described in male and female rats. To this aim, F344 rats aged 11 weeks at arrival were selected in order to have sexually mature male and female animals during the study, and they were orally administered with OTA for one or three weeks. The present study was designed to reproduce a study previously published (Arbillaga et al., 2008) carried out only in male F344 rats. In the aforementioned study, OTA (0.50 mg/kg bw) was administered to animals for 7 and 21 days. This dose was selected as it is slightly higher than the one used in several carcinogenicity studies (Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998; Mantle et al., 2005; Son et al., 2003). In this experiment, the same experimental design but including female rats was used. Moreover, a lower dose (0.21 mg/kg bw) was included at the longer exposure time (21 days) to gain more insights at histopathological level. This dose was selected as it was the highest dose tested in the carcinogenicity study carried out by the NTP (NTP, 1989).

Our results showed mild toxic effects in both sexes that were more pronounced in male rats than in female rats. In terms of general toxicity, a significant decrease of body weight was observed in males, after 14 days of treatment with the highest dose. The presence of blood cells in urine and a slight proteinuria, indicative of kidney damage, were also more pronounced in males than in females treated with the highest dose. Regarding histopathology, kidney degenerative lesions, mainly in proximal tubules, were determined in a time-dependent manner in both sexes but a different pattern has been observed: after 7 days of treatment with 0.50 mg/kg bw, the number of animals with glomerulonephritis, tubulonephrosis or alterations in the collecting duct was higher in the male group than in the female one. Moreover, the intensity of the lesions was also higher in males than in females. But after 21 days of treatment, sex differences had disappeared. Other authors have also found kidney toxicity in male rats of different strains (Wistar, F344, Sprague Dawley), with short-term treatments (2–4 weeks) of 0.20–0.50 mg OTA/kg bw (Abdu et al., 2011; Arbillaga et al., 2008; Cariddi et al., 2016; Ciarcia et al., 2016; El-Haleem et al., 2016; Malekinejad et al., 2011; Palabiyik et al., 2013; Taniai et al., 2012; Rached et al., 2007). Rached et al., 2007 determined a No Observed Adverse Effect Level (NOAEL) of 0.021 mg/kg bw after a 13-week study in male F344 rats. But, apart from the carcinogenic life-span studies carried out with F344 rats (Boorman et al., 1992; NTP, 1989) or with the Dark-Agouti and Lewis strains (Castegnaro et al., 1998; Son et al., 2003), very few studies have been performed with rats of both sexes. In weaning Wistar rats fed with an OTA contaminated diet (5 ppm) during 90 days, Munro et al. (1974) did not found differences in the OTA toxicity that could be related to sex. In male and female F344 rats administered with 1 mg OTA/kg bw for 7 consecutive days, Rasonyi et al. (1999) found several kidney lesions that were similar in appearance and location in both sexes but more frequent and severe in males than in females. In this study, the characteristic OTA-induced karyomegalic cells have been observed in both sexes after 21 days of treatment. This in agreement with Boorman et al. (1992) and the NTP study (1989) where karyomegaly could be observed with the same incidence in both sexes. As expected, mycotoxin administration resulted in a time- and dose-dependent increase of OTA in plasma, kidney, and liver of male and female rats. In addition, a clear correlation between plasma and tissues concentration was observed, being the OTA plasma concentration approximately 10-fold the concentration found in liver and kidney, which were very similar between them. This is in agreement with previous dose-repeated toxicity studies carried out in male F344 rats orally administered with OTA for 6–21 days (Abbas et al., 2013; Arbillaga et al., 2008). Regarding sex differences in OTA accumulation,

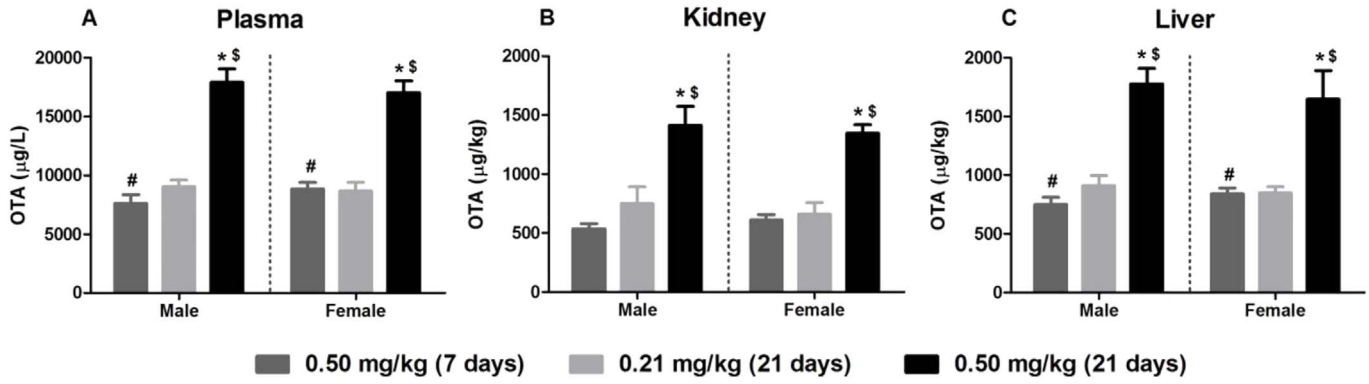

Fig. 4. HPLC-FLD OTA quantification in plasma (A), kidney (B) and liver (C) samples obtained from male and female F344 rats treated with a daily oral dose of OTA for 7 (0.50 mg/kg bw) or 21 days (0.21 or 0.50 mg/kg bw). Differences were considered statistically significant when *p*-value < 0.05 (Mann-Whitney *U* test). Within each sex, comparisons were made between the doses (0.21 and 0.50 mg/kg bw after 21 days) (*), and between the duration of the treatment (7 and 21 days, 0.50 mg/kg bw) ($). Under each condition, comparisons between both sexes have been studied (#). For comparison with other studies, raw data in μg/L or μg/kg as well as on a molar basis can be found in table 5 supplementary data.

they could only be weakly detected in plasma and liver after 7 days of administration. However, after a long exposure, no significant differences between males and females could be identified in any matrix. This is in agreement with Tozlovanu et al. (2012) who did not find differences in OTA concentrations between sexes in kidney and liver of male and female Dark-Agouti rats consuming OTA contaminated wheat for 28 days, and with the predictions of the kinetic model developed by Vettorazzi et al. (2009, 2011). However, Mor et al. (2014) in a longer study (24 weeks) found higher levels of OTA in plasma, kidney and liver in females than in males.

The expression of four different transporter families was studied in terms of mRNA levels. Firstly, basal levels were analyzed in control animals (Fig. 7A and C). Sex differences in Oat2 (M < F), Oat5 (M < F), Bcrp (M > F) and Oatp1 (M > F) transporters were observed. The strongest differences were found in the apical transporter Oat2, with mRNA level 8- or 9-fold higher in females than in males. Oatp1 and Bcrp mRNA levels were higher in males with no expression of the former in females. These are the most consistent findings and confirm previous results in F344 rats (Kwekel et al., 2013; Pastor et al., 2016).

The expression of transporters was also studied after repeated administration of 0.50 mg/kg bw for 7 or 21 days (Fig. 7). To our knowledge, this is the first study analyzing gene expression modulation of OTA transporters in animals of different sexes after repeated treatment. In agreement with our results, some toxicogenomic studies have demonstrated a predominant downregulation of kidney transporters in male F344 rats treated for 7 and 21 days at similar doses: 0.30 mg/kg (Marin-Kuan et al., 2006) or 0.50 mg/kg (Arbillaga et al., 2008). Both studies confirmed Oat1 (Slc22a6) and Oatp1 (Slco1a1) downregulation. Arbillaga et al. (2008) also determined a decreased expression of Oat2 (Slc22a7), Oat5 (Slc22a9), Oat8 (Slc22a19) and Pept2 (Slc15a2) after 21 days. Similarly, in male Wistar rats treated with 0.50 mg/kg bw for 10 days, Zlender et al. (2009) found a downregulation of Oat1, Oat2, Oat3 and Oat5. Regarding Mrp2 expression, our results showed an increase of its mRNA level in both sexes after 21 days. In male F344 rats under similar conditions, a decrease in mRNA level was found by Arbillaga et al. (2008) and Marin-Kuan et al. (2006) but in Elker male rats treated with 0.21 mg/kg bw after 1, 3, 7 and 14 days, Stemmer et al. (2007) also found an increase in Mrp2 expression, in agreement with our results.

Overall, our results support the fact that OTA modulates the gene expression of different renal proteins involved in its transport. Indeed,

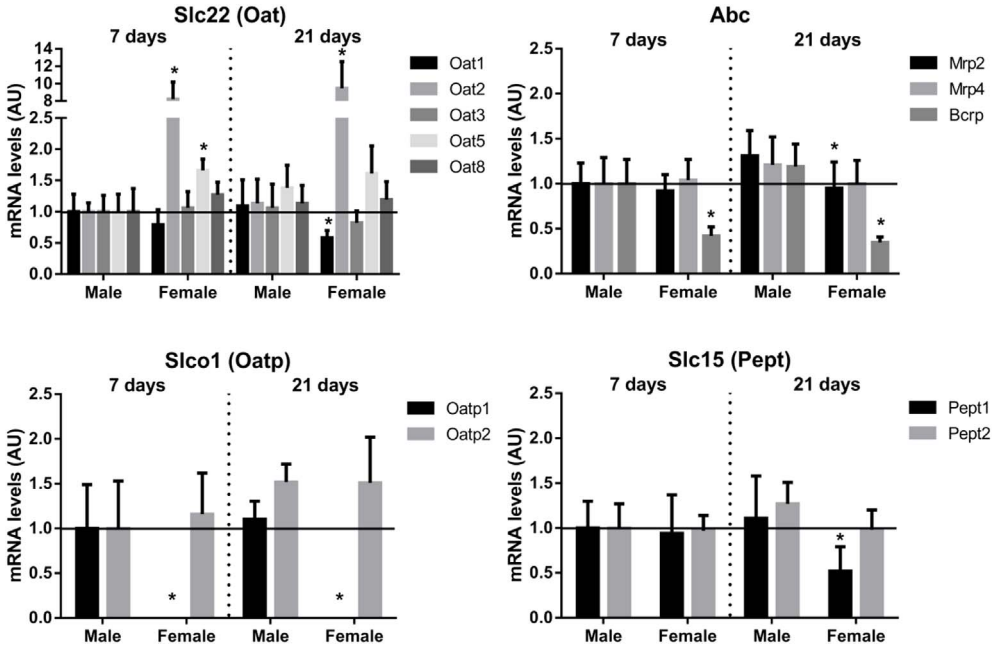

Fig. 5. Kidney transporter basal expression in F344 rats treated with a daily oral dose of vehicle ($NaHCO_3$) for 7 or 21 days. 7-day male control rats were used as calibrator group (1 AU mRNA, shown as a black line). For each transporter, within each treatment time, comparisons between sexes were studied (*). For each transporter, within each sex, comparisons between 7 and 21 days were studied ($). Expression changes were considered statistically significant when *p*-value < 0.05 (Mann-Whitney *U* test). *AU: arbitrary units of mRNA level.*

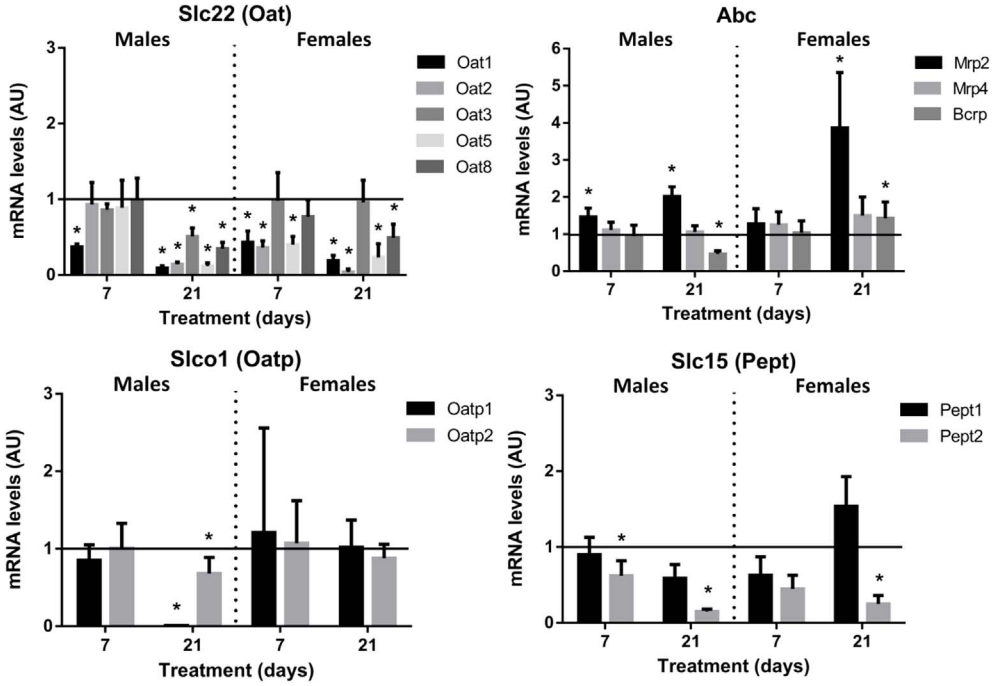

Fig. 6. Transporter expression changes in kidney samples obtained from male and female F344 rats treated with a daily oral dose of vehicle ($NaHCO_3$) or OTA (0.50 mg/kg bw) for 7 or 21 days. For each sex and timepoint, its own control group has been used as calibrator group (1 AU mRNA, shown as a black line). Within each sex, gene expression of each transporter after 7 or 21 days of OTA treatment was compared to its control. Expression changes were considered statistically significant (*) when *p*-value < 0.05 (Mann-Whitney *U* test). *AU: arbitrary units of mRNA level.*

our study shows, for the first time, that OTA modulation shows important sex-dependent differences (Fig. 6). Some of the most important distinctive aspects are related with a different direction of modulation in each sex (Bcrp), absence of modulation in one sex but downregulation in the other (Oat3) or absence of basal expression in one sex (Fig. 5) but high modulation by OTA in the other sex (such as Oatp1).

Moreover, changes in expression of the different transporters do not appear at the same timepoint in each sex. This influence of the time of OTA exposure appears to be especially important for Oat transporters. In females, the Oat downregulation observed after 21 days was already observed at 7 days; while in males the strong downregulation found at 21 days was not present after 7 days (except for Oat1). The different effect of time in each sex, can also be also observed in a study carried out after a single oral dose of OTA (0.50 mg/kg bw), where an increase in the expression of some Oat transporters (mainly Oat2) was observed in male rats after 24 h of OTA exposure, while in females, downregulation was already the predominant effect since the beginning (Pastor et al., 2016). In another study carried out in Wistar male rats (Zlender et al., 2009) a dual effect in Oat gene expression was observed depending on the dose administered (upregulation at low doses, down regulation at high dose). Thus, time- and dose-dependent effects appear to be crucial for the correct interpretation of sex differences at kidney transporter level after OTA exposure.

However, despite the gene expression sex differences observed in the kidney transporters at individual level, it is difficult to conclude how the final balance (Fig. 7) would influence OTA toxicokinetics in both sexes, but it does not seem to support a higher accumulation of OTA in male rats as proposed by Mally (2012). Indeed, this is in accordance with the similar OTA levels measured in the kidney in males and females (Fig. 4).

On the other hand, and as many kidney transporters are regulated by sex hormones, hormone levels were quantified in the present study, A clear dose- and time-dependent decrease of progesterone was determined in females. Progesterone and testosterone, in males, and estradiol, in females, were weakly increased after 21 days. T_3 and T_4 levels were not modified in any sex after mycotoxin administration. The role of OTA as endocrine disruptor has been slightly studied and the whole range of experimental systems, doses or time of exposure make hard to draw a conclusion about mycotoxin-induced effect on hormone regulation. In agreement with our results, an *in vitro* study using the JEG-3 cell line (human placenta cells), determined an increase dose- and time-dependent of progesterone production (Woo et al., 2013). Frizzell et al. (2013) using H295R cell line as steroidogenesis model, showed an increase of estradiol production, but no effect in testosterone and progesterone levels. Regarding *in vivo* studies, testosterone level increased in testis of male Wistar rats, after 3 weeks of OTA treatment (289 μg/kg bw each 48 h) (Gharbi et al., 1993). However, Kumar et al. (2011) and Hassan et al. (2010) observed a testosterone reduction after 1 or 6 months of administration of 4 or 1 ppm OTA in feed, respectively. Finally, Mor et al. (2014), observed no changes in testosterone level in 16-week-old male rats that were fed with 20 g of contaminated diet (5 mg/kg in feed) for 24 weeks.

Regarding transporter regulation by hormones, our results indicate that progesterone might be involved in OTA-induced changes. As mentioned before, this hormone increases Oat1, Oat2 and Oat5, does not affect Oat3 and diminishes Bcrp and Pept1 expressions (Breljak et al., 2010; Ljubojevic et al., 2004, 2007; Watanabe et al., 2006; Wu et al., 2013). In agreement with this, the decrease of Oat1, Oat2, Oat5, the increase of Bcrp and Pept1 (no statistically significant) and the no effect on Oat3 expression observed in females is correlated with the progesterone decrease. With respect to the males, they have lower basal progesterone levels than females and only a tendency to its increase was observed after OTA treatment together with a slight tendency to testosterone increase. Therefore, it is difficult to correlate the variation in both hormone levels with the effect in transport expression in males.

In conclusion, after 7 days of treatment, histopathological findings were indicative of a more pronounced effect in male kidneys than in female ones with a tendency to be equal in both sexes over time. No significant differences in plasma, liver or kidney OTA concentrations were found between both sexes, with a plasma concentration that was approximately ten fold higher than liver and kidney concentrations at both time points. Analysing individual transporter expression, sex differences were determined both at basal level and after OTA treatment. Downregulation was the general OTA-induced effect. Oats response was slower in males than females and Oat3 did not show any expression change in females. Oatp1 was solely and strongly downregulated in males after 21 days. Finally, an opposite effect was observed in Bcrp after 21 days: its expression decreased in males but increased in

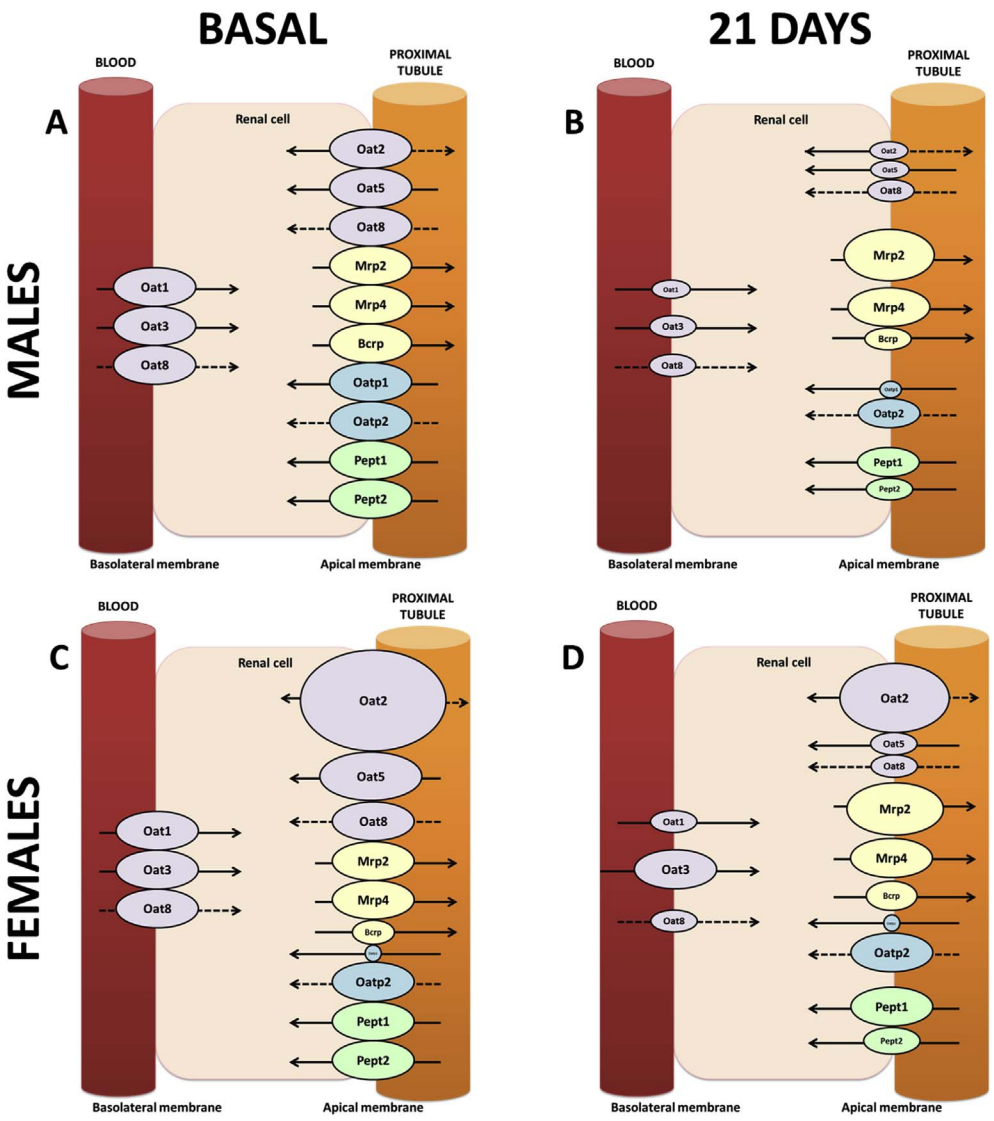

Fig. 7. (modified from Pastor et al., 2016). Illustration of the differential gene expression of the kidney transporters evaluated. The figure shows the localization and direction of transport of each kidney transporter. Each transporter family is represented with a different color: Slc22 (Oat1, Oat2, Oat3, Oat5 and Oat8), Abc (Mrp2, Mrp4 and Bcrp), Slco1 (Oatp1,Oatp2) and Slc15 (Pept1 and Pept2). The dimension of the circle illustrates the relative mRNA levels shown in Fig. 5 and 6. A) Basal males (selected as reference for the comparisons). B) Males treated with OTA (0.50 mg/kg) bw for 21 days (changes are relative to A). C) Basal females (changes are relative to A). D) Females treated with OTA (0.50 mg/kg) bw for 21 days (changes are relative to C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

females. Moreover, a progesterone dose- and time-dependent decrease was observed in females, and it might be involved in Oats, Bcrp and Pept1 regulation. Overall, our results indicate that 1) OTA treatment modulates kidney transporters gene expression in a sex-dependent manner; 2) the influence of the time of OTA exposure in each sex seems crucial when evaluating kidney transporters in both sexes and 3) despite the sex-differences observed, the final balance of the influence of the different transporters gene expression in OTA toxicokinetics at renal cell level, does not seem to support a higher accumulation of OTA in male kidneys.

Acknowledgments

This work was supported by the University of Navarra through the PIUNA Project ¨Efecto cancerígeno de la ocratoxina A: influencia del sexo en el mecanismo de acción" [PIUNA 2012]. Laura Pastor thanks the "Asociación de Amigos" (University of Navarra) for the grant received.

The authors are grateful to Ms. Celia Goñi for her kind help with the biochemical analysis.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2017.11.003.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2017.11.003.

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