Phenolic acid protects of renal damage induced by ochratoxin A in a 28-days-oral treatment in rats

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ABSTRACT

The present study aimed to characterize the chlorogenic acid (ChlA) capacity to reverse the toxic effects induced by ochratoxin A (OTA) in a subacute toxicity test in rats. Male Wistar rats were fed orally by gavage for 28 days with OTA (0.4 mg/kg bw/day), ChlA (5 mg/kg bw/day) or the combination OTA (0.4 mg/kg bw/day) + ChlA (5 mg/kg bw/day). No deaths, no decrease in feed intake or body weight in any experimental group were recorded. The negative control group and the animals treated with ChlA alone showed no changes in any parameters evaluated. In OTA-treated group significant changes such as decrease in urine volume, proteinuria, occult blood, increase in serum creatinine values; decrease in absolute and relative kidney weight and characteristics histopathological lesions that indicated kidney damage were observed. However, limited effect on oxidative stress parameters were detected in kidneys of OTA-treated group. Animals treated with the combination OTA + ChlA were showed as negative control group in the evaluation of several parameters of toxicity. In conclusion, ChlA, at given concentration, improved biochemical parameters altered in urine and serum and pathological damages in kidneys induced by OTA exposure, showing a good protective activity, but not by an apparent antioxidant mechanism.

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1. Introduction

Ochratoxin A (OTA) is one of the most abundant mycotoxins produced by some fungal species of *Aspergillus* and *Penicillium*. It contaminates food and foodstuff and widespread in cereals, grains, coffee beans, dried grapes, juice, wine, beer and bread due to improper storage conditions (WHO, 2001). This mycotoxin has nephrotoxic, hepatotoxic, immunotoxic, mutagenic and genotoxic

properties (Pfohl-Leszkowicz and Manderville, 2007; Mantle et al., 2010).

Diverse studies have demonstrated that OTA induced nephrotoxicity and hepatotoxicity through oxidative DNA damage *in vitro* (Schaaf et al., 2002; Kamp et al., 2005) and *in vivo* (Meki and Hussein, 2001; Di Giacomo et al., 2007). Several physical, chemical and biological methods have been developed to suppress or reduce the toxic action of mycotoxins, improve food safety and minimize economic losses (Zinedine et al., 2007; Battacone et al., 2010). Recently, attention has been focused on natural antioxidant constituents. Polyphenols are secondary metabolism products of plants and constitute one of the most numerous and widely distributed groups of natural antioxidants in the plant kingdom (Wollgast and Anklam, 2000). In a recent study we have demonstrated that the addition of polyphenols (luteolin, chlorogenic or caffeic acids) during 24 h in Vero cells or lymphocytes cultures treated with OTA reduced the toxic effects caused by this mycotoxin with good protective effect. In that study we also have evaluated the effect of these polyphenols on DNA damage induced by OTA
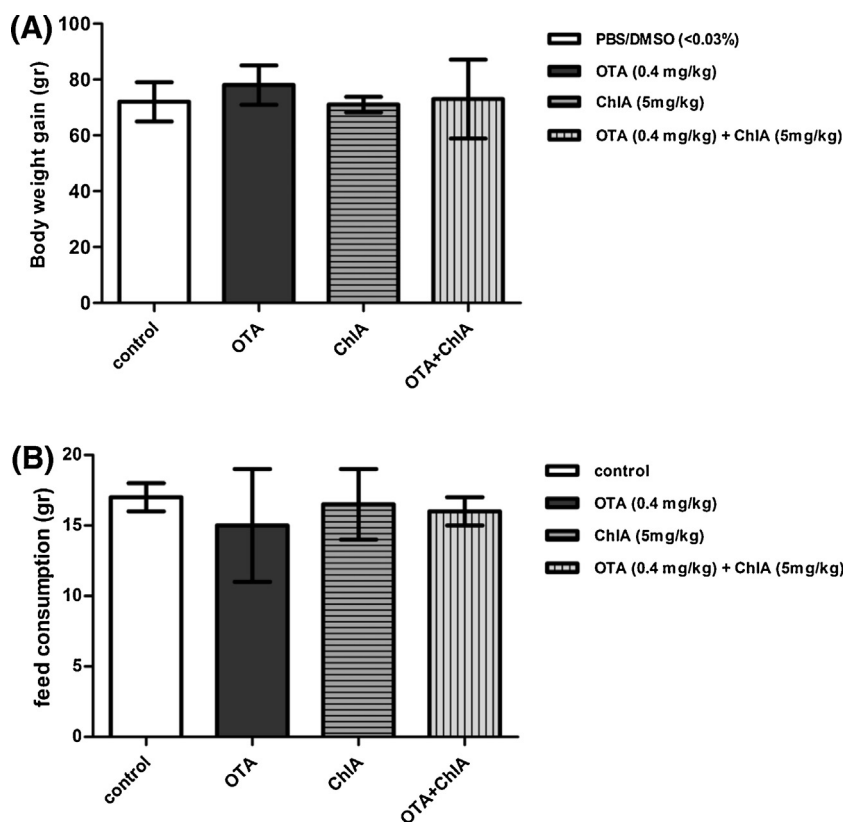
Fig. 1. (A) Body weight gain and (B) feed consumption of Wistar rats in the 28 days of treatment with OTA, chlorogenic acid or combination of OTA + chlorogenic acid. Each value represents means ± SD. ChlA: chlorogenic acid; DMSO: dimethylsulfoxide. Statistical test: ANOVA and Tukey's Test.

on blood cells and bone marrow cells of Balb/c mice and observed that only chlorogenic acid was able to reduce OTA-induced DNA damage on both cells type (Cariddi et al., 2015). Chlorogenic acid is a potent antioxidant found in the group of phenolic acids, which are the most abundant, accounting for 60% of total polyphenols in the diet, approximately 1 g/day (Manach et al., 2004; Meng et al., 2013) that appear to be an effective alternative for reducing the toxic effects caused by this mycotoxin.

Based on our previous results, the present study aimed to demonstrate a potential beneficial effect of chlorogenic acid in a subacute toxicity study in rats exposed to OTA. For that, parameters on general toxicity and biochemical biomarkers were measured and histopathological examinations were performed.

2. Materials and methods

2.1. Reagents

Ochratoxin A was purchased from BioPure Technology (UK); dimethylsulfoxide (DMSO) was obtained from Merck (Germany) and chlorogenic acid (≥95%) was purchased from Sigma St. Louis (USA). Commercially available standard reagents were used for the determination of urea in serum (Uremia), urea in urine (Ureasa), creatinine in urine and serum (Creatinina Directa), total proteins (Proteínas Totales AA), glucose in serum (Glucosa líquida AA), ALAT (Transaminasas 200 GPT) and ALP (Fosfatasa Alcalina Optimizada) all purchased from Wiener Lab (Rosario, Argentina). OTA and chlorogenic acid were dissolved in DMSO/PBS (solvent concentration not exceeding 0.03%).

2.2. Animals and treatments

Young male Wistar rats aged 10–12 weeks old (weighing 150–200 g), supplied from Bioterio Central of the Universidad Nacional de Río Cuarto, were maintained in a temperature and humidity controlled room, with 12 h light-dark cycles and were allowed food and water *ad libitum*. All experimental procedures were conducted in accordance with recent legislation. This study was approved by the Comité de Ética de la Investigación Científica (COEDI), Universidad Nacional de Río Cuarto.

Table 1
Absolute and relative (% body weight) weights of the organs of Wistar rats after oral treatment for 28 days with OTA, chlorogenic acid or the combination of both.

		Control	OTA (0.4 mg/kg)	ChlA (5 mg/kg)	OTA (0.4 mg/kg) + ChlA (5 mg/kg)
Kidney	Absolute weight (mg)	1363 ± 47.87	1150 ± 57.74***	1350 ± 57.74	1333 ± 57.74##
	Relative weight (% body weight)	0.527 ± 0.01	0.493 ± 0.02**	0.564 ± 0.03	0.6113 ± 0.05##
Liver	Absolute weight (mg)	17400 ± 424.3	15050 ± 212.1	16000 ± 1131	12200 ± 989.9**
	Relative weight (% body weight)	6.640 ± 0.15	6.455 ± 0.09	6.705 ± 0.88	5.850 ± 0.38
Heart	Absolute weight (mg)	1100 ± 141.4	1150 ± 70.71	1200 ± 141.4	1000 ± 282.8
	Relative weight (% body weight)	0.420 ± 0.05	0.493 ± 0.03	0.503 ± 0.08	0.472 ± 0.06

ChlA: chlorogenic acid, **$p < 0.01$, ***$p < 0.001$ statistically significant differences respect to negative control; ##$p < 0.01$ statistically significant differences respect to OTA-treated group (ANOVA and Tukey's Test).
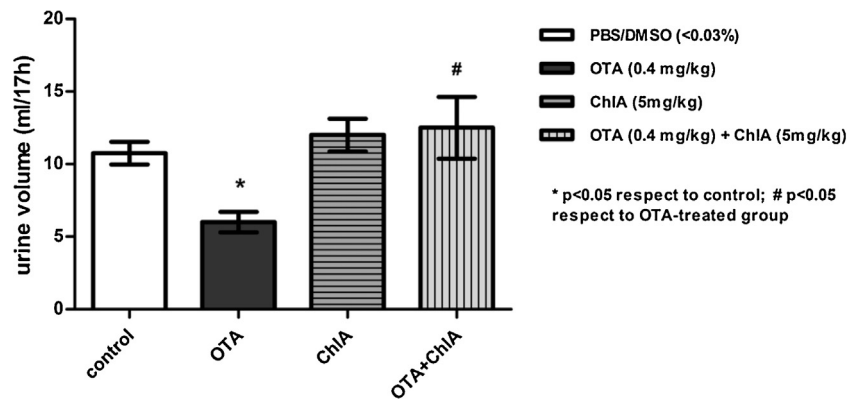
Fig. 2. Urine volume of Wistar rats in the 21 days (third week of the experiment) of treatment with OTA, chlorogenic acid or combination of OTA + chlorogenic acid. Each value represents means ± SD. ChlA: chlorogenic acid; DMSO: dimethylsulfoxide. Statistical test: ANOVA and Tukey's Test.

Animals were separated into groups of 4 animals per group and were treated individually by gavage during the entire experimental period of 28 days as follows:

1 With a dose of DMSO/PBS as vehicle control group (0.2 ml/day) containing the same amount of DMSO or PBS as the test groups.
2 With a dose of OTA (0.4 mg/kg bw/day).
3 With a dose of chlorogenic acid (5 mg/kg bw/day).
4 With combination of OTA (0.4 mg/kg bw/day) and chlorogenic acid (5 mg/kg bw/day).

The selection of OTA and the polyphenol doses was based on other *in vivo* studies in rats (Dortant et al., 2001; Palabiyik et al., 2013) and on previous study (Cariddi et al., 2015).

2.3. General toxicity parameters

The survival and clinical signs were checked once daily. Feed consumption was recorded daily and body weight once a week.

2.4. Biochemical parameters in urine and serum

In the third week of the experiment, animals were placed in metabolic cages for 17 h, then urine was collected and urinary volume was recorded. Total protein, urea and creatinine were measured by standard reagents in a spectrophotometer (Spectrum SP-2000 VIS Spectrophotometer, Shanghai, China) at 540 and 510 nm, respectively. The pH was measured in a pH meter (Hanna

Instruments, Argentina); glucose, ketone, bilirubin, occult blood, nitrite and urobilinogen were measured with reactive strips (Urine Strip) from Wiener Lab (Rosario, Argentina). Clearance rates (C) for urea and creatinine were calculated (C = urinary volume × urinary concentration/blood concentration). After collection of urine, blood samples were collected from the tail vein and serum samples were prepared by centrifugation. The following biochemical parameters: urea, creatinine, alanine aminotransferase (ALAT), alkaline phosphatase (ALP) and glucose were measured by standard reagents in a spectrophotometer at 540, 510, 520 and 505 nm, respectively.

2.5. Histopathology

On day 29 of experiment, animals were decapitated without being anesthetized and liver, kidneys and heart were dissected immediately. The macroscopic external features (weight, size and color) of these organs were registered. Parts of the organs were stored at −20 °C until further analysis. Other parts were preserved in 10% buffered formaldehyde (pH 7.4). These samples were cut at 4 μm thickness and subjected to haematoxylin/eosin (H&E) staining for microscopic histological examination under 400 × magnifications. Photomicrographs were taken with a Zeiss Axiostar plus microscope using an Electronic Eyepiece camera with MIAS (Micro Image Analysis Software 2008, v 2.2) software and a Canon Power Shot G5 camera (Canon Inc., Japan). To evaluate the level of damages following exposure to OTA, chlorogenic acid or a combination of both, the evaluation criteria were as follows: 0 for

Table 2
Protective effects of chlorogenic acid on OTA-induced histopathological damages.

	Control	OTA (0.4 mg/kg bw/day)	ChlA (5 mg/kg bw/day)	OTA (0.4 mg/kg bw/day) + ChlA (5 mg/kg bw/day)
Kidney				
TN	0.0 ± 0.0	3.0 ± 0.0***	0.5 ± 0.7	0.0 ± 0.0###
ED	0.5 ± 0.7	3.0 ± 0.0***	1.0 ± 0.0	1.0 ± 0.0##
H	0.0 ± 0.0	2.5 ± 0.7***	1.0 ± 0.0	0.0 ± 0.0###
Liver				
V	0.0 ± 0.0	1.5 ± 0.7	0.5 ± 0.7	0.8 ± 0.3
MGC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CP	0.5 ± 0.7	1.5 ± 0.7	0.5 ± 0.7	0.8 ± 0.3
Heart				
HY	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
I	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
E	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

ChlA: chlorogenic acid, TN: tubular nephrosis, ED: eosinophilic hyaline droplets, H: hemorrhage, V: vacuolation, MCG: multinucleated giant cells, CP: biliary/oval cell proliferation, I: inflammation, Hy: hyperemia, I: inflammation, E: edema.
0: not detectable lesion, 1: mild changes, 2: moderate changes, 3: severe changes. ***p < 0.001 statistically significant differences respect to negative control; ##p < 0.01; ### p < 0.001 statistically significant differences respect to OTA-treated group (ANOVA and Tukey's Test).

(A)

(B)
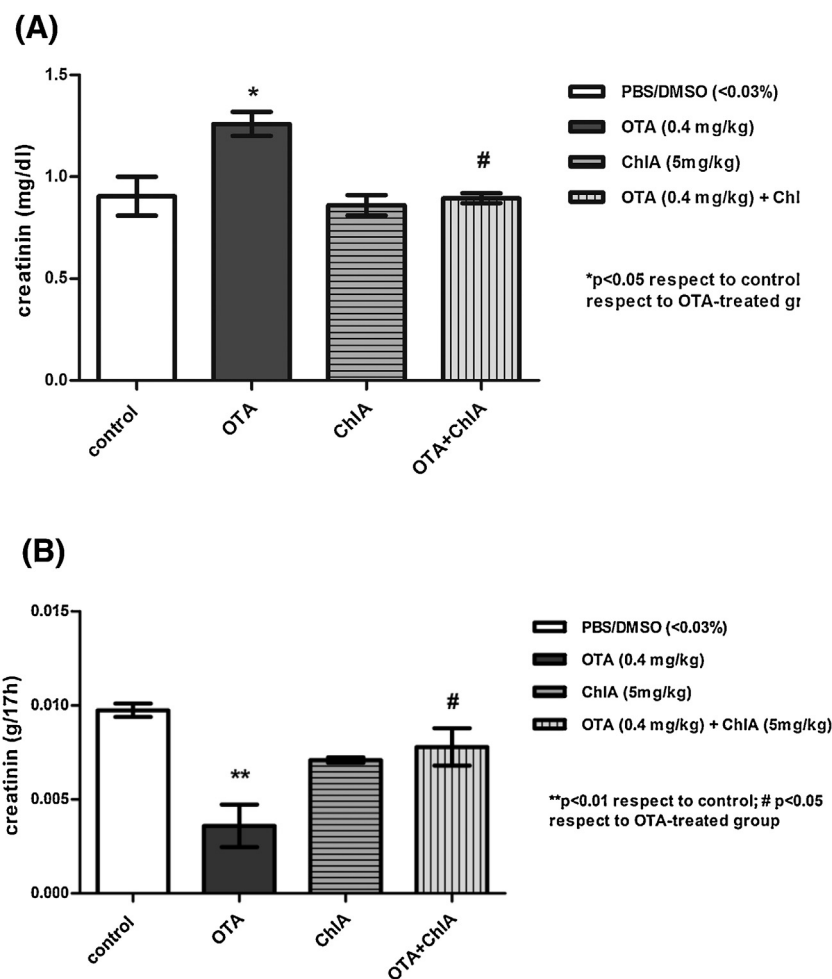
Fig. 3. (A) Serum creatinine levels and (B) urine creatinine levels of Wistar rats in the 21 days (third week of the experiment) of treatment with OTA, chlorogenic acid or combination of OTA + chlorogenic acid. Each value represents means ± SD. ChlA: chlorogenic acid; DMSO: dimethylsulfoxide. Statistical test: ANOVA and Tukey's Test.

no detectable lesion, 1 for mild changes, 2 for moderate changes, and 3 for severe changes.

2.6. Oxidative stress parameters

Frozen kidney tissues were thawed at room temperature and homogenates (10%) were prepared in chilled 0.05 M potassium phosphate buffer, pH 7.4. Thiobarbituric acid reactive substances (TBARs) concentrations, expressed as nmol of malondialdehyde (MDA)/g of tissue were measured spectrophotometrically at 532 nm in kidney homogenates using the method of Marcincak et al. (2003). The concentrations were determined using standard curves of MDA. Superoxide dismutase activity was assayed spectrophotometrically in the supernatants of kidney homogenates by the method of Misra and Fridovich (1972). One unit of enzymatic activity has been defined as the amount of enzyme which causes 50% inhibition of auto oxidation of epinephrine.

2.7. Statistical analysis

All the values obtained in the assays were expressed as averages with standard deviations. The data were evaluated using GraphPad Prism version 5.00.288 (San Diego, USA, 2007) and compared with one way analysis of variance (ANOVA) and the Tukey multiple comparison tests. The differences were considered to be statistically significant at $p < 0.05$.

3. Results

During the 28 days of the *in vivo* assay no deaths in any experimental group were recorded. Respect to clinical signs of toxicity, it was observed that animals group treated with OTA showed aggressiveness against tampering. This observation was also recorded for animals treated with the combination OTA + chlorogenic acid which showed aggressive but less than the group treated with OTA. In addition, one of the four animals of OTA group had generalized edema. Animals from negative control group and those treated with chlorogenic acid alone, showed no clinical signs of toxicity. No decrease in feed consumption was observed and the body weight gain showed no significant differences between the animals from control group and those of treated groups (Fig. 1A and B).

Significant changes in several parameters such as urine volume, creatinine, total protein and occult blood, were detected in the urine samples of animals from OTA-treated group. Urine volume was significantly lower in the OTA-treated group compared to the volume of urine of animals belonging to the other groups. Urine volume in the animals treated with the combination OTA + chlorogenic acid was similar of the negative control group and significantly higher than the amount of urine of the OTA-treated group ($p < 0.05$) (Fig. 2). High protein levels were also observed in urine samples of OTA-treated group and of animals treated with the combination of OTA + chlorogenic acid compared to the negative control group and the treated group with chlorogenic acid alone. As expected, creatinine levels in serum were significantly increased and remark-
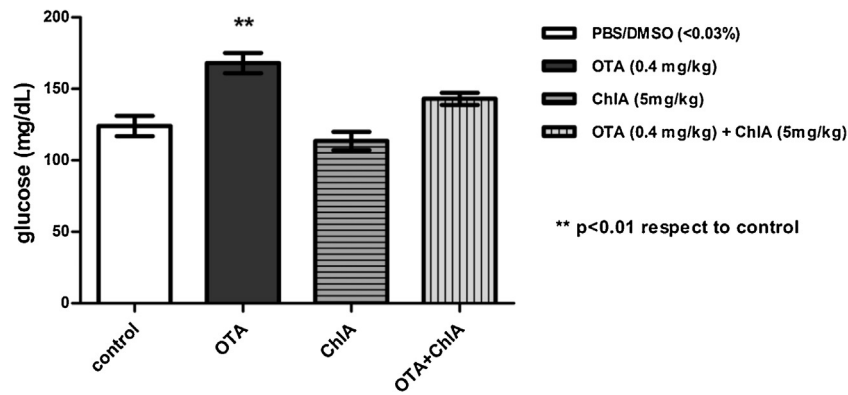
Fig. 4. Serum glucose levels of Wistar rats in the 21 days (third week of the experiment) of treatment with OTA, chlorogenic acid or combination of OTA + chlorogenic acid. Each value represents means ± SD. ChlA: chlorogenic acid; DMSO: dimethylsulfoxide. Statistical test: ANOVA and Tukey's Test.

ably decreased in urine from OTA-treated group respect to other groups ($p < 0.05$), indicating kidney dysfunction. The creatinine levels in serum and urine from animals treated with combination of OTA + chlorogenic acid were found similar to negative control group (Fig. 3A and B). Clearance of creatinine was significantly decreased. However, the values of urea in urine and serum were within normal limits in all groups. Occult blood was found only in the urine samples of OTA-treated group. No occult blood was found in the urine samples of animals treated with the combination of OTA + chlorogenic acid group. Other biochemical parameters determined in urine (pH, glucose, bilirubin, ketones, urobilinogen and nitrite) showed no abnormalities in either group. With respect to others biochemical parameters determined in serum ALP and ALAT levels showed no abnormalities in either group, however, high glucose values were observed in OTA-treated group respect to negative control group ($p < 0.01$). In the group of animals treated with the combination of OTA + chlorogenic acid a slight decrease of glucose levels was observed but without statistically significant differences respect to OTA-treated group (Fig. 4).

Moreover, a significant decrease in the absolute ($p < 0.001$) and relative ($p < 0.01$) kidneys weight in the group of animals treated

with OTA respect to the negative control group and the other treated groups was observed.

The kidneys weight in the group of animals treated with chlorogenic acid alone and with combination of OTA + chlorogenic acid remained normal without differences with the negative control group. A statistically significant differences in the kidneys of animals treated with combination of OTA+ chlorogenic acid respect to OTA-treated group was found ($p < 0.01$) (Table 1).

Regarding to absolute liver weight, a significant decrease in the group treated with the combination OTA + chlorogenic acid ($p < 0.01$ respect to negative control group) was observed; however, the relative weight remained normal. The absolute and relative heart weight in the treated groups showed no significant difference with respect to the negative control group (Table 1).

Histopathological examination of the kidneys of OTA-treated group revealed characteristic lesions of nephrotoxicity: tubular nephrosis, Bowman spaces and lumens with presence of protein moieties as eosinophilic hyaline droplets, and areas of interstitial hemorrhage. In contrast, the kidneys of the negative control group and animals treated with chlorogenic acid alone showed a normal appearance (Table 2 and Fig. 5). It is noteworthy that the histopathology of kidney of animals treated with the combination
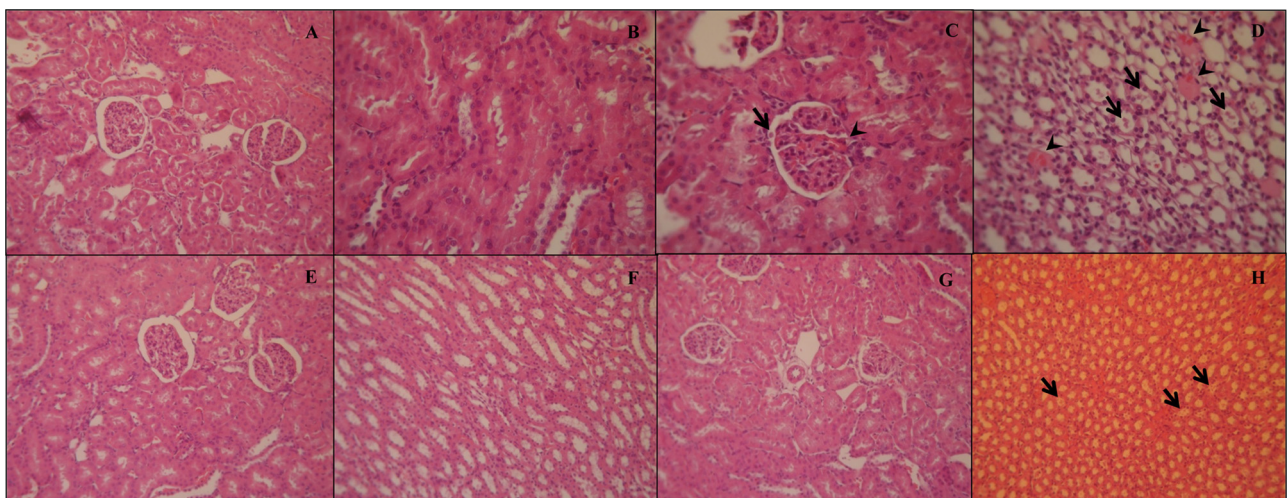
Fig. 5. Histopathology of representative kidney lesions of Wistar rats treated orally during 28 days with OTA, chlorogenic acid or combination of both. (A) Appearance of the normal kidney in the control group (10×). (B) Appearance of kidney belonging to OTA-treated group (20×). Tubular nephrosis is observed. (C) and (D) Histopathological appearance of kidney belonging to OTA-treated group (20×). Arrows indicate presence of protein moieties as eosinophilic hyaline droplets in Bowman spaces and lumens; and arrowheads indicate areas of interstitial hemorrhage. (E) Appearance of kidney belonging to animals treated with chlorogenic acid alone (10×) which is observed similar to the control. (F), (G) and (H) Histopathological appearance of kidney belonging to animals treated with combination of OTA + chlorogenic acid (10×). In figure (F) tubular nephrosis is not observed, figure (G) is similar to control and in figure (H) presence of protein moieties as eosinophilic hyaline droplets was observed. Figures (A), (B), (C), (E) and (G) are in renal cortex; figures (D), (F) and (H) are in renal medulla.
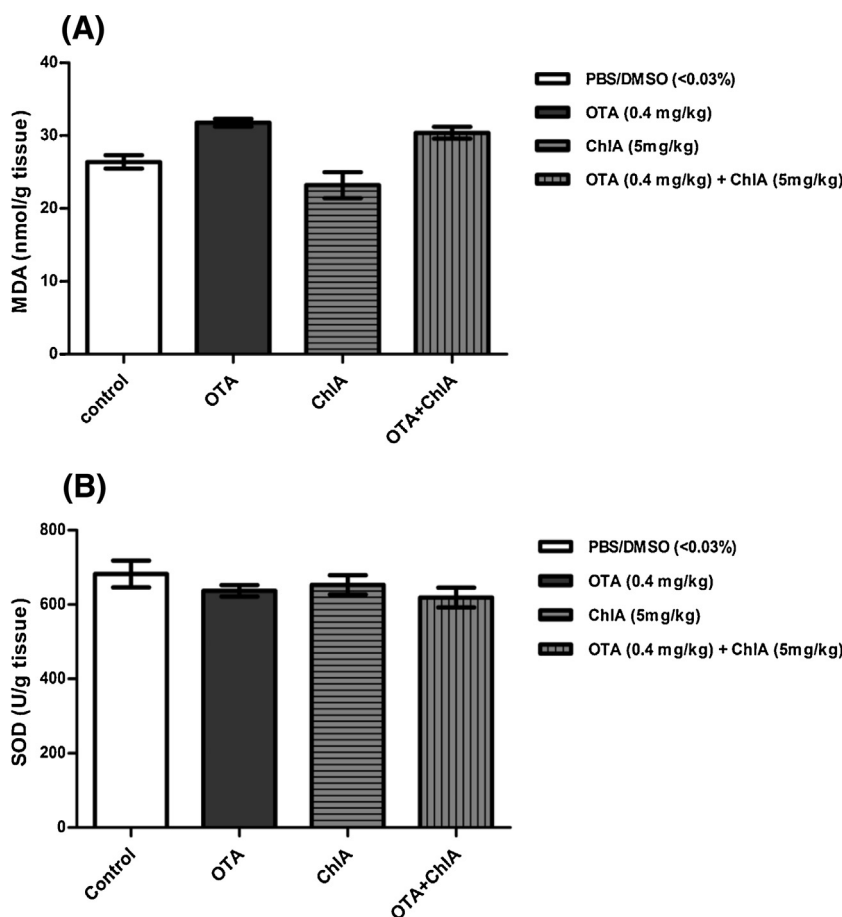
Fig. 6. (A) Malondialdehyde (MDA) values and (B) superoxide dismutase (SOD) values of kidneys of Wistar rats treated orally during 28 days with OTA, chlorogenic acid or combination of both. Each value represents means ± SD. ChlA: chlorogenic acid; DMSO: dimethylsulfoxide. Statistical test: ANOVA and Tukey's Test.

OTA + chlorogenic acid was similar to the negative control without characteristic lesions of toxicity. Significant differences respects to OTA-treated group were found ($p < 0.001$ and $p < 0.01$) (Table 2 and Fig. 5).

The histopathology of liver showed some lesions as vacuolation or biliary/oval cell proliferation mainly in OTA-treated group however, no significant differences respect to negative control neither to others treated groups were found (Table 2). Histopathological examination of the heart showed no signs of toxicity in any treatment group (Table 2).

No statistically significant changes were observed in the levels of MDA and SOD in the kidneys of OTA-treated group compared to the other treated groups (Fig. 6A and B).

4. Discussion

In the present study, we evaluated whether the oral treatment with chlorogenic acid could protect against toxic damage caused by OTA exposure in rats. This study found that the kidney was morphologically and physiologically the most affected after oral treatment for 28 days with OTA (0.4 mg/kg bw/day) which is consistent with other studies (Dortant et al., 2001; Malekinejad et al., 2011; Qi et al., 2014) and confirms the nephrotoxic effects of OTA. In OTA-treated animals we observed the excretion of low volumes of urine which is a characteristic of the acute ochratoxicosis, which it presents with polydipsia and polyuria to end in anuria (Perusia and Rodríguez, 2001). In addition, we observed edema in one of animal of this group, which could be a consequence of loss of the ability of kidneys

to remove excess fluid and electrolytes. However, the urine volume in animals treated with combination of OTA + chlorogenic acid was similar to control group, showing the protective effect of this polyphenol on renal damage induced by OTA. High concentration of protein was also observed in urine samples both of OTA-treated group and animals treated with combination of OTA + chlorogenic acid. Proteinuria is an early sign of kidney damage caused by OTA both in man as in animals (Perusia and Rodríguez, 2001; Hsieh et al., 2004) and in this case no protective effect of chlorogenic acid was observed. OTA-treated group showed a significant increase of serum creatinine. It is known that high levels of serum creatinine are indicative of an impairment of kidney function. Our results are in agreement with those of Dortant et al. (2001) and Malekinejad et al. (2011), who reported high levels of serum creatinine in rats treated orally with OTA during 28 days. The levels of serum creatinine in animals treated with the combination of OTA + chlorogenic acid were normal, indicating a protective effect of chlorogenic acid on toxic damage induced by OTA. Occult blood also was found in urine of OTA-treated group. The presence of blood in urine suggests kidney damage. No blood was found in the urine of animals treated with the combination OTA + chlorogenic acid group, which would indicate a protective effect of chlorogenic acid. It was also observed high glucose levels in serum of OTA-treated group. It is known that OTA impairs proximal tubular functions in the kidney, and causes glucosuria (Anzai et al., 2010). In this case no protective effect of chlorogenic acid was observed. The histopathological examination of kidneys from OTA-treated group showed characteristic lesions of nephrotoxicity which is consistent with others investigations (Dortant et al., 2001; Malekinejad et al., 2011; Qi

et al., 2014) and with biochemical parameters measured in urine in the present work. It notes that the histopathology of kidneys from animals treated with the combination OTA + chlorogenic acid was almost similar to the negative control group, although, according to the biochemical parameters measured in urine, the presence of proteins was detected.

Although the mechanisms of OTA-induced toxic effects have not yet been clearly elucidated several studies have demonstrated that after cell treatment with OTA there are an increase of ROS levels and a reduction of antioxidant enzymes (SOD, GSH) which leads to induce a wide range of lesions in cell membranes, proteins and DNA (Baudrimont et al., 1997; Abdel-Wahhab, 2000; Abdel-Wahhab et al., 2005; Liu et al., 2012). In the present study we measured two parameters of oxidative stress (MDA and SOD) in the kidneys of animals treated with OTA, chlorogenic acid and the combination of both. MDA is a lipid peroxidation product formed by the action of reactive oxygen species (ROS) on polyunsaturated lipids and SOD is an important marker of oxidative stress. We not found changes in the levels of parameters measured in all treated groups which leads us to believe that the damage induced by OTA in the kidneys of treated animals were probably not caused by oxidative stress. Our results are in agreement with those of some authors that have reported that oxidative stress will not play a major role in the toxicity induced by OTA (Pfohl-Leszkowicz and Manderville, 2012; Hibi et al., 2013; Qi et al., 2014). Some reports indicate that diseases caused by OTA in the kidney may be associated with a potential carcinogenicity. The modes of action proposed include DNA-adduct formation, cell proliferation, modulation of apoptosis, and alteration of gene expression (Mally and Dekant, 2009). Given that in a previous study we confirmed that OTA induced DNA damage in mice and chlorogenic acid exerted a protective effect on this action (Cariddi et al., 2015); it could be that this compound has exerted their protective effect of renal damage in the same way, but not through an antioxidant mechanism. Currently studies are underway to elucidate this.

In conclusion, chlorogenic acid at given concentration, improved some biochemical parameters altered in urine and serum and pathological damages in kidneys induced by OTA exposure, showing a good protective activity. To our knowledge, this work is the first to report the ability of chlorogenic acid to reduce the renal damage induced by OTA in an oral treatment, which could contribute to the prevention of ochratoxicosis.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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