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Effect of Ethanol and Red Wine on Ochratoxin A-Induced Experimental Acute Nephrotoxicity

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Ochratoxin A (OTA), is a nephrotoxic mycotoxin present in wine, which is nephrotoxic in humans. Our working hypothesis is that natural substances in wine may counteract OTA toxicity. Thirty-six rats were randomized to OTA dissolved in saline, red wine, or 13.5% ethanol or to OTA-free wine, ethanol, or saline. OTA (289 μg/kg of body weight/48 h) was administered by gastric gavage for 2 weeks. Serum creatinine, tubular enzymuria, renal lipohydroperoxides (LOOH), reduced (GSH) and oxidized (GSSG) glutathione, and renal superoxide dismutase activity (SOD) were determined in renal tissue. OTA alone produced significant increases in renal lipoperoxides and significant decreases in SOD and GSH/GSSG ratio. In red wine or ethanol, OTA was less nephrotoxic, reducing oxidative damage as revealed by LOOH. In OTA−wine and OTA−ethanol groups, SOD activity was higher than in the OTA-treated one, suggesting that both ethanol and nonalcoholic fractions may preserve antioxidant reserve. GSH/GSSG ratio was significantly preserved only in the OTA−wine group and not in OTA−ethanol. Red wine may exert a protective effect against OTA nephrotoxicity by limiting oxidative damage. The ostensible protection afforded by ethanol deserves further investigation.

KEYWORDS: Ochratoxin A; red wine; nephrotoxicity; rat; ethanol; lipid peroxidation; glutathione; superoxide dismutase activity

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin produced by several species of *Aspergillus* and *Penicillium*, which contaminates animal feed and human food, including cereal and grain products, when stored under improper conditions (*1*). It has been identified in blood, bile, and urine of humans and animals after consumption of contaminated food (*2*, *3*) also in Western countries (*4*, *5*). OTA and its metabolites have nephrotoxic and carcinogenic activity in the kidney (*6*, *7*). In humans it has been associated with so-called Balkan endemic nephropathy and it might be implicated in the onset of urinary tract tumors (*8*−*11*). The proximal tubule of the kidney is a primary target site in OTA-induced nephrotoxicity (*12*, *13*): OTA is transported into tubular cells and is eliminated by the classic organic anion transport system (*14*). The exact mechanism of OTA nephrotoxicity is not completely understood: it may act as a genotoxic carcinogen and/or its nephrotoxicity may be due to an indirect mechanism, such as induction of cytotoxicity or increased cellular proliferation. However, in recent years several studies have stressed the importance of oxidative stress. OTA induces oxidative damage in cultured cells and in experimental animals (*15*, *16*); moreover, antioxidant administration is able to reduce its nephrotoxicity (*17*).

Recently, significant levels of OTA contamination have also been detected in coffee (*18*), beer, grape juice, wine, and musts (*19*−*23*), alarming the public and health authorities (*24*).

However, no epidemiological studies have demonstrated that all these beverages, especially red wine, can induce nephrotoxicity. Thus, the goals of this study were to check whether equivalent doses of OTA administered daily by oral route can induce nephrotoxicity in rats, and if so, to determine the effects of ethanol and wine on this toxicity. Our working hypothesis was that if OTA was shown to induce nephrotoxicity under these conditions, this may be due to an inflammatory process, which could be prevented by natural phenolic compounds. We chose red wine, because it contains the highest concentrations of

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Table 1. Analytical Composition of Red Wine (Barbera d'Asti, 2001) Employed in the Experiment

density	0.993
alcohol (%)	13.44
tot. extract (g/L)	26.8
tot. acidity (g/L)	7.20
vol. acidity (g/L)	0.80
tot. anthocyanins (mg/L)	149
tot. flavonoids (mg/L)	1150
delphinidin (%)	6.05
cyanidin (%)	3.83
petunidin (%)	7.54
peonidin (%)	6.90
malvidin (%)	42.42
acetate Ant. %	23.48
cinnamate Ant. %	9.78
caffeil tartaric acid (mg/L)	102.4
p-cumaril tartaric acid (mg/L)	29.9
trans-ferulil tartaric acid (mg/L)	5.1
caffeic acid (mg/L)	7.3
trans-resveratrol (mg/L)	3.2
cis-resveratrol (mg/L)	3
tartaric acid (g/L)	2.81
malic acid (g/L)	0.04
shikimic acid (g/L)	0.021
lactic acid (g/L)	1.64
quercetin glucuronide (mg/L)	10.1
quercetin glucoside (mg/L)	3.1
kampherol glucuronide (mg/L)	16.9
kampherol glucoside (mg/L)	1.4
catechins (mg/L)	35.6
epicatechin (mg/L)	13.0

polyphenols found in wines exhibiting antioxidant (*25*), anticancer (*26*, *27*), and antiinflammatory (*28*) properties. On the basis of these observations, we decided to ascertain whether substances found in red wine may counteract OTA-induced nephrotoxicity.

MATERIALS AND METHODS

Experimental Protocol. Thirty-six male Wistar rats weighing 180−200 g were housed in single metabolic cages at constant room temperature and humidity under a controlled light/dark cycle. Rats were fed a standard chow diet and had free access to drinking water. All experiments were performed in accordance to guidelines of our local ethical committee. Rats were randomized to one of the six following groups of treatment:

OTA-Treated Rats (Group I). From day 1 to day 14, each rat was treated with OTA (Sigma−Aldrich, St. Louis, MO) at a dose of 289 μg/kg of body weight every other day by gastric gavage, as previously reported (*29*). OTA was dissolved in 0.1 M $NaHCO_3$, pH 7.4 and then diluted in 0.5 mL of saline.

Rats Treated with Red Wine Containing OTA (Group II). From day 1 to day 14, each rat was treated with OTA as reported above but diluted in 0.5 mL of an OTA-free red wine (Barbera d'Asti, 13.5% by volume). Its analytical composition was determined by HPLC and spectrophotometry as previously described (*30*−*32*). The results are reported in **Table 1**.

Rats Treated with Hydroalcoholic Solution Containing OTA (Group III). From day 1 to day 14, each rat was treated with OTA as reported above but diluted in 0.5 mL of a hydroalcoholic solution containing ethanol at the same concentration as in red wine (13.5% by volume).

Rats Treated with Red Wine without OTA (Group IV). From day 1 to day 14, each rat was treated with 0.5 mL of the OTA-free red wine (Barbera d'Asti, 13.5% by volume).

Rats Treated with Hydroalcoholic Solution without OTA (Group V). From day 1 to day 14, each rat was treated with 0.5 mL of a hydroalcoholic solution containing 13.5% ethanol.

Control Rats Treated with Saline without OTA (Group VI). From day 1 to day 14, each rat was treated with 0.5 mL of saline.

Urine samples (24 h) were collected daily to determine creatininuria and enzymuria (alanine aminopeptidase, AAP, and *N*-acetylglucosaminidase, NAG). At the end of the experimental period, blood samples were collected in anesthetized rats to determine serum creatinine. The serum was separated by centrifugation. Plasma and urinary creatinine were measured by a highly sensitive colorimetric method (BioAssay Systems, Hayward, CA). Finally, animals were sacrificed by an anesthetic overdose and the kidneys were removed.

Some specimens were fixed by immersion in 3.3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 2 h at 4 °C, washed in 0.1 M phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated through an ascending series of ethanols, and embedded in Araldite. Ultrathin sections were cut with a Reichert Ultracut R ultramicrotome (Leica, Wien, Austria), stained with uranyl acetate and lead citrate, and then observed with a JEOL CX100 transmission electron microscope (JEOL, Tokyo, Japan).

Kidney aliquots were collected to determine the renal content of lipid hydroperoxides (LOOH colorimetric assays); of total (GSH) and oxidized (GSSG) glutathione (both assays use an enzymatic recycling method with glutathione reductase), employing commercially available kits (Cayman Chemical, Ann Arbor, MI); and superoxide dismutase (SOD) activity (spectrophotometric assay), employing commercially available kits (Oxis Health Products, Inc.). Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. To eliminate any interference caused by hydrogen peroxide or endogenous ferric ions in the sample, lipid hydroperoxides were extracted into deoxygenated chloroform and methanol, and the extract was directly used in the assay. This procedure provided a sensitive and reliable assay for lipid peroxidation. The assay was performed by following the kit-included protocol.

Samples to determine GSH and SOD were prepared as follows: prior to dissection, kidneys were perfused with a phosphate-buffered saline (PBS) solution, pH 7.4, containing 0.16 mg/mL heparin to remove any red blood cells and clots. After dissection, the samples for the determination of GSH were homogenized in 5 mL of cold buffer (50 mM phosphate, pH 6−7, containing 1 mM EDTA) per gram of tissue and centrifuged at 10 000 rpm for 15 min at 4 °C; the supernatant was removed and stored on ice. Before GSH assaying, the supernatant was deproteinated and then stored at −20 °C. The kidney samples for the determination of SOD activity were homogenized after the perfusion, in 5 mL of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose per gram of tissue and centrifuged at 1500 rpm for 5 min at 4 °C; the supernatant was removed and stored at −80 °C.

Finally, the assays to determine GSH and SOD activity were performed by using the provided solutions and following the kit-included protocols.

All chemicals were obtained from Fluka Chemie (Buchs, Switzerland) and Sigma Chemical Co. (St. Louis, MO). The OTA-free red wine (Barbera d'Asti 2001) has been kindly supplied by Istituto Sperimentale per l'Enologia (Experimental Institute for Enology), Asti, Italy. This wine was supplied with detailed analytical data (Table 1) and guaranteed as OTA-free.

Statistical Analysis. ANOVA followed by Neuman-Keuls' test was used and the null hypothesis was rejected when $p < 0.05$. Results were expressed as mean value ± standard deviation.

RESULTS

Our results confirmed that OTA induces renal injury as revealed by enhanced S-creatinine (0.83 ± 0.11 versus 0.46 ± 0.12 mg/dL, $p < 0.05$) and elevated enzymuria (**Figure 1**). Renal function impairment was related to oxidative injury: in group I, renal LOOH was 4.04 ± 2.30 versus 1.43 ± 1.09 pmol/mg of protein in controls (groups IV−VI; $p < 0.05$) (**Figure 2**), and the GSH/GSSG ratio was 1.08 ± 0.56 versus 3.32 ± 1.25 (**Figure 3**). Renal SOD activity (**Figure 4**) was also reduced by OTA (40.64 ± 9.84 versus 51.49 ± 6.42 units/mg of protein, $p < 0.05$). Data obtained in OTA−wine and OTA−ethanol groups demonstrated a significant reduction in renal lipid

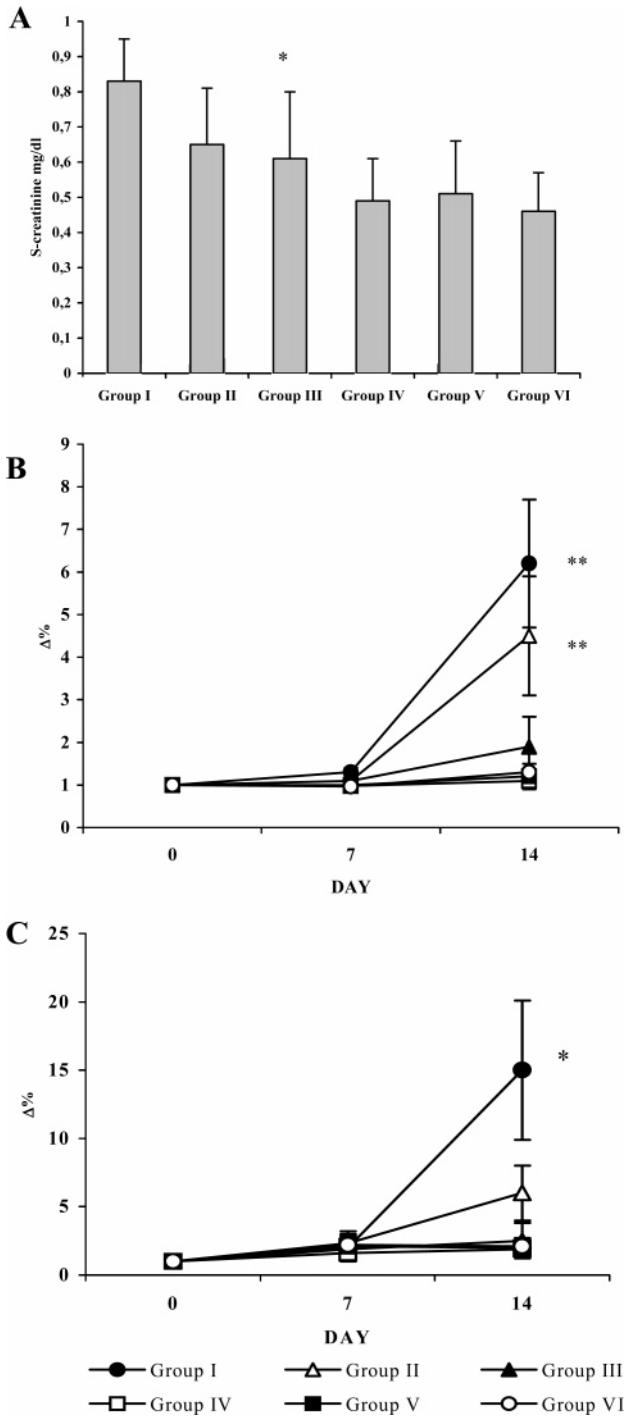

Figure 1. Serum creatinine (**A**) at the end of experimental period and enzymuria time course in rats of each group (**B**, AAP; **C**, NAG). Results are expressed as mean ± SD (*p < 0.05 vs groups II–VI; **p < 0.05 vs groups III–VI).

peroxidation (2.44 ± 1.33 and 2.01 ± 1.26 pmol/mg of protein, respectively; p < 0.05 versus OTA) (**Figure 2**) and an increase in SOD activity (47.54 ± 9.55 and 46.07 ± 6.92units/mg of protein, p < 0.05 versus OTA). However, the GSH/GSSG ratio was preserved in OTA−wine-treated rats but not in OTA−ethanol-treated ones (2.89 ± 1.12, p < 0.05 versus OTA and OTA−ethanol) (**Figure 3**). This lower oxidative injury was also confirmed by normal S-creatinine.

Ultrastructural analysis confirmed these results: tubular brush border, disrupted in rats treated with OTA (**Figure 5B**), was totally preserved in the OTA−ethanol group (**Figure 5C**) and

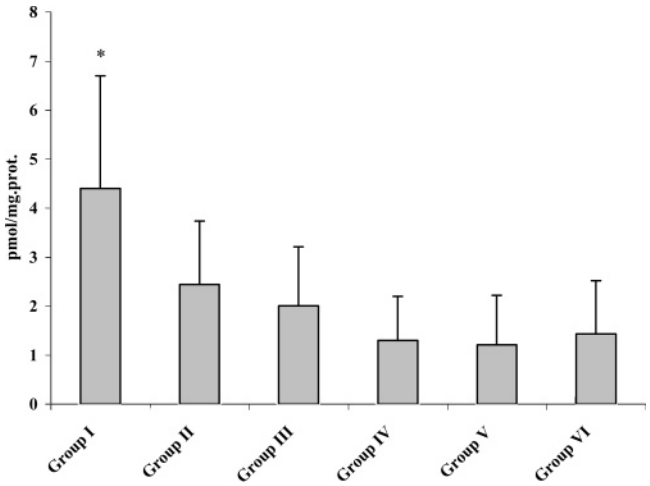

Figure 2. Renal content in LOOH in rats of each experimental group. Results are expressed as mean ± SD. OTA-induced lipid peroxidation is prevented by ethanol and wine (*p < 0.05 vs groups II– VI).

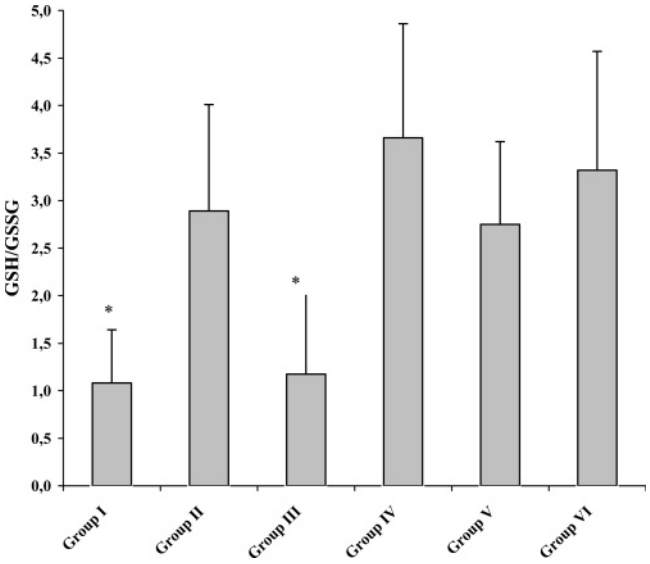

Figure 3. GSH/GSSG ratio in renal tissue. Results are expressed as mean ± SD. GSH/GSSG ratio was preserved only in OTA–wine-treated rats (*p < 0.05 vs groups II and IV–VI).

was partially preserved in OTA−wine-treated rats (**Figure 5D**). Significant differences versus controls were not detected in groups V and VI.

DISCUSSION

Our results confirmed that OTA induces acute nephrotoxicity, although its extent was limited by the relatively low doses administered. It induces oxidative damage with enhanced lipid peroxidation, which is one of the most important mechanisms of action in OTA acute nephrotoxicity. Previous studies (*33*) have shown that OTA is able to chelate Fe^{3+}, forming a complex that is promptly reduced by NADPH−cytochrome P450 reductase in OTA−Fe^{2+}, which, in the presence of oxygen, initiates lipid peroxidation. Moreover, Baudrimont and co-workers (*29*) reported that reactive oxygen species were involved in OTA-induced nephrotoxicity. OTA is known to affect especially the proximal tubule (*34*), where cells are very sensitive to oxidative damage: in our study we found the typical alterations, including enhanced tubular enzymuria, brush border injury, and reduced renal function, associated with enhanced lipid peroxidation and reduced tissue antioxidant enzymes.

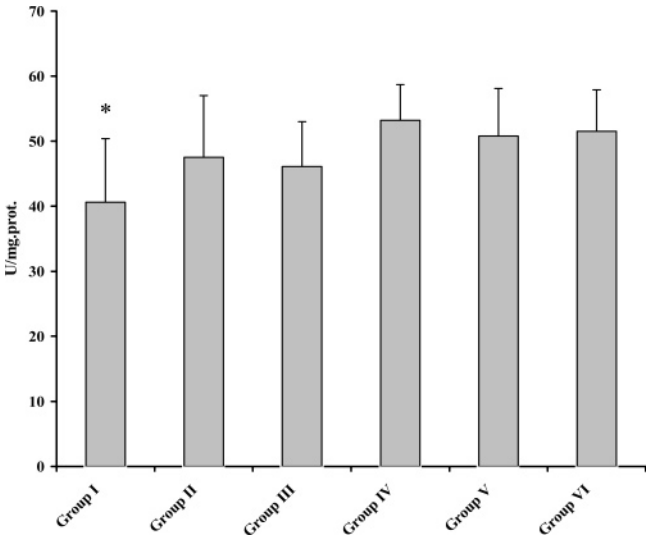

Figure 4. Renal SOD activity. Results are expressed as mean ± SD. SOD was significantly reduced in OTA-treated rats (*$p < 0.05$ vs groups II–VI).

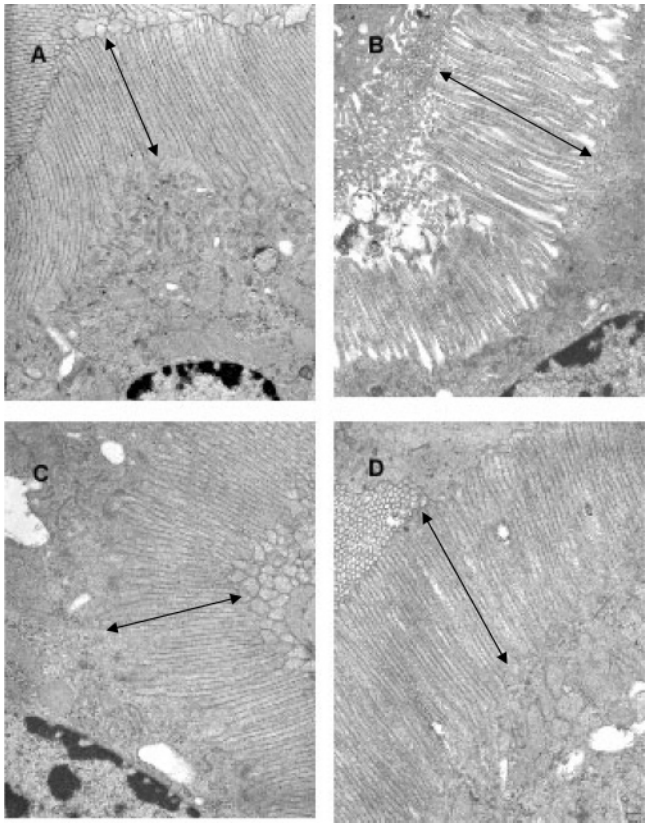

Figure 5. Electron microscopy of rat kidney tubular cells. Brush border, regularly present in controls (**A**), was disrupted in rats treated with OTA (**B**). Brush border was totally preserved in the OTA–ethanol group (**C**) and was partially preserved in the OTA–wine group (**D**). Magnification 10000×. Arrows indicates the brush border.

Our main observation is that ethanol intake (dissolved in water, 13.5% by volume) prevents OTA-induced nephrotoxicity when it is administered either as red wine or mixed with water. Prevention was assessed in terms of both enzymuria and ultrastructural findings. The most likely explanation of this effect is that consumption of ethanol induces various biotransformation enzymes, especially the CYP450-dependent ones. Indeed, it has been demonstrated that pretreatment with a CYP450 inducer protected rats against OTA nephrotoxicity, whereas cobalt−protoporphyrin IX pretreatment, which decreases CYP450 levels, exacerbated OTA nephrotoxicity (*35*). Furthermore, ethanol was shown to induce CYP450 in the liver (*36*) and in the kidneys (*37*). Thus, it is conceivable that OTA toxic activity is reduced by ethanol-enhanced CYP450 activity. The observation that wine appears to be less protective than an ethanol solution of the same titer was unexpected. The OTA−ethanol group showed significantly lower enzymuria than the OTA group. In OTA−wine-treated rats, AAP urinary excretion was significantly lower than in OTA-treated rats; however, no difference in NAG urinary levels was found between OTA- and OTA−wine-treated animals. At present we have no clear explanation for this result. The lower protective activity of OTA-containing red wine could be explained by direct interference of its nonalcoholic components with CYP450, as previously shown in the liver (*38*) and the kidneys (*39*). It is noteworthy that at least four natural compounds present in the red wine used in our experiments (quercetin, naringenin, catechin, and resveratrol) have shown inhibitory effects on CYP450 2B1 activity in rat liver microsomes (*40*). Thus, a decrease of ethanol CYP450-dependent enzyme induction by some components of the red wine is a likely hypothesis.

Another important finding is that the GSH/GSSG ratio was preserved in OTA−wine-treated rats, whereas it was not by ethanol solution (nonsignificant difference). This clearly indicates that some wine components, such as polyphenols, add their antioxidant effects to the preservation process by counteracting peroxidation damage. The fact that OTA−wine-treated rats exhibit quite normal GSH/GSSG ratios indicates that the various physiological antioxidant defenses are preserved. This finding is in accordance with the previous report by Rodrigo et al. (*41*), who recently demonstrated that red wine antioxidant polyphenols could increase the GSH/GSSG ratio in the kidney. Moreover, Schaaf et al. (*15*) reported that in vitro covalent binding of OTA to GSH could inactivate the mycotoxin and promote its excretion from the tubular cell, suggesting that GSH levels could play a pivotal role in limiting OTA nephrotoxicity. Thus, polyphenols may limit the amount of GSH needed to counteract the peroxidation process.

In conclusion, our study has demonstrated for the first time that OTA acute nephrotoxicity may be limited by both ethanol and nonalcoholic red wine components. We suggest that OTA contamination of alcoholic beverages should be taken into consideration when the nephrotoxicity of this mycotoxin is assessed. However, the fact that ethanol appears to be more protective than red wine warrants further experiments. Our results did not show the well-known toxic effects of ethanol. This could be due to the fact that we used a low ethanol dose for a brief period. In our opinion, the toxic action of ethanol could become evident in subchronic or chronic toxicity studies.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GSH, total glutathione; GSSG, oxidized glutathione; HPLC, high-pressure liquid chromatography; LOOH, lipohydroperoxides; OTA, ochratoxin A; SOD, superoxide dismutase.

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