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# Recombinant Mitochondrial Manganese Containing Superoxide Dismutase Protects Against Ochratoxin A-Induced Nephrotoxicity

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## ABSTRACT

Ochratoxin A (OTA) is a natural mycotoxin, involved in the development of important human and animal diseases. In this work we have studied the role of oxidative stress in the development of OTA nephrotoxicity and the effect of a new recombinant mitochondrial manganese containing superoxide dismutase (rMnSOD) to prevent kidney damage induced by OTA. Blood pressure, glomerular filtration rate and renal histology were analyzed in control rats and in OTA treated rats. In addition, lipid peroxidation, catalase and superoxide dismutase productions were measured. Our data showed that animals treated with OTA presented hypertension and reduction of glomerular filtration rate (GFR). These effects are most probably related to an increase in the reactive oxygen species (ROS) productions. In fact, we have shown that treatment with rMnSOD restored the levels of blood pressure and GFR simultaneously. Moreover, we have noted that OTA induced alteration on glomerular and tubular degeneration and interstitial infiltrates and that use of rMnSOD combined with OTA prevent this renal histological damage confirming the potential therapeutic role in the treatment of rMnSOD OTA nephrotoxicity. J. Cell. Biochem. 117: 1352–1358, 2016. 

**KEY WORDS:** OCHRATOXIN A; NEPHROTOXICITY; rMnSOD; OXIDATIVE STRESS

Ochratoxin A (OTA), (*R*)-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1*H*-2-benzopyran-7-yl) carbonyl] -L-phenylalanine, is a natural mycotoxin produced by *Aspergillus* and *Penicillium* species with important implications for animal and human health [Krogh, 1976; Bozić et al., 1995]. It is found in cereals, green coffee, dried fruits and meat products, resulting in continuous exposure of the human and animal population to OTA [Duarte et al., 2010]. For this reason several methods of detoxification in human and animal have been developed to reduce the toxic effects of OTA [Creppy et al., 1998; Costa et al., 2007; Fusi et al., 2010; Palabiyik et al., 2013].

OTA is absorbed from the gastrointestinal tract and in the small intestine. It is distributed into the liver, muscle, fat and it is accumulated into the kidney, where specific transporters may be

Roberto Ciarcia and Sara Damiano equally contributed to the work.

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involved in its cellular uptake [Costa et al., 2007]. OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic and immunotoxic in several species of animals [Ribelin et al., 1978; Ringot et al., 2006; Palabiyik et al., 2013] and in humans, it has been associated to Balkan endemic nephropathy (BEN) [Boorman et al., 1992; Ringot et al., 2006] although an exact mechanism of action is still unclear. Several mechanisms of OTA toxicity have been proposed, as well as the contribution of OTA to cause an imbalance between oxidant and antioxidant parameters [Bui-Klimke and Wu, 2015]. It is interesting to note that OTA increases the expression of nitric oxide synthase [Wang et al., 2008] enzyme responsible for the production of nitric oxide (NO) that is known to react with oxygen anion superoxide ($O_2^-$) to form the pro-oxidant peroxynitrite. Moreover, it is well demonstrated the contribution of OTA to increase the reactive oxygen species (ROS) production, which may lead to oxidative stress and molecular damage that contribute to the pathogenesis of chronic diseases [Castegnaro et al., 1998; Grollman and Jelaković, 2007].

The mechanism of chronic nephrotoxic diseases has been previously investigated by our research group. In particular, we have demonstrated, in a rat animal model of hypertension induced by cyclosporine, that the increase in ROS production, in particular the $O_2^-$, was linked to a strong reduction of glomerular filtration rate (GFR) [Ciarcia et al., 2015]. Since the reduction of antioxidants expression has been shown to be related to an increase of oxidative damage [Klaunig et al., 1998; Marin-Kuan et al., 2011] which contribute to OTA toxicity.

Several antioxidants were tested, in vivo and in vitro, to counteract the negative effects of ROS generated by OTA, such as vitamin E [Fusi et al., 2010], catechins [Costa et al., 2007] and licopene [Palabiyik et al., 2013] showing that they may partially protect against OTA induced nephrotoxicity and oxidative stress. In an our previous paper we have demonstrated the protective effect of rMnSOD on renal function of CsA hypertensive rats after chronic administration [Damiano et al., 2013].

In this study we have evaluated the effect of rMnSOD (a family member of SOD codified by different genes localized exclusively in the mitochondrial matrix [Okado-Matsumoto and Fridovich, 2001; Ridnour et al., 2004; Mancini et al., 2006] and able to alter the growth rate and invasiveness of cancer cells [Mancini et al., 2008] on in vivo glomerular filtration rate (GFR), on ROS production and in histological damage in rat animal model treated for 14 days with OTA. Moreover we have evaluated the relationship between OTA, ROS production and the renal damage.

## MATERIALS AND METHODS

### CHEMICALS AND REAGENTS

The rats were obtained from Harlan Laboratories Srl (San Pietro al Natisone, Udine, Italy); Ochratoxin A (OTA), Inulin and all the reagents were obtained from Sigma–Aldrich (Milan, Italy), isoflurane (Isotec 4, Ohmeda) by Albamedical (Naples, Italy) and rMnSOD was obtained by Molecular Biology and Viral Oncology Department of Experimental Oncology, Istituto Nazionale Tumori "Fondazione G. Pascale", IRCCS (Naples, Italy) [Mancini et al., 2006], Catalase and SOD activity assays kits were purchased by Caymac Chemical Company.

### ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Italian Ministry of Health (Permit Number: 2015-261-PR).

### EXPERIMENTAL PROTOCOL

Experiments were performed on a total of 48 adult male Sprague Dawley rats weighing 120–150 g. They were housed under constant environmental conditions (temperature 22 °C and a 12 h light–dark cycle). Animals were fed a standard diet; food and water were given ad libitum. The rats, randomly divided into four groups, were treated orally for 14 days as follows: Group 1 (Control group), 12 rats treated with a corresponding amount of saline solution (including 10% DMSO); Group 2 (rMnSOD group), 12 rats treated with rMnSOD (10 μg/kg); Group 3 (OTA group): 12 rats treated with OTA (0,5 mg/Kg bw) dissolved in 10% DMSO and then scaled to required volume with corn oil; Group 4 (rMnSOD + OTA), 12 rats treated with rMnSOD (10 μg /kg) plus OTA (0,5 mg/Kg bw). The dose and length of rMnSOD and OTA administration was chosen according to previous experiments [Palabiyik et al., 2013; Damiano et al., 2013]. Blood Pressure (BP) was recorded in anaesthetized rats, during the clearance experiments, through the carotid artery using a blood pressure recorder (Pressure Monitor BP-1, Word Precision Instruments).

### CLEARANCE OF INULIN

GFR was measured at the end of the experimental treatment. The rats were anaesthetized with 2% isoflurane (Isotec 4, Ohmeda), placed on a thermo-regulated table (37 °C) and prepared for renal clearance evaluation. In brief, the right carotid artery was catheterized to monitor BP through a blood pressure recorder (BP1 by WPI) and to take blood samples for inulin concentration measurements. The left jugular vein was cannulated with polyethylene PE-50 tubing and used for intravenous infusion via a syringe pump (Braun, Melsungen) of 0.74 mg/100 g bw/min of inulin from 10% saline solution. The surgical procedure also included bladder catheterization with PE-205 tubing. After a 60 min equilibration period, the first of four 30 min urine collections began. Arterial blood samples (100 μl) were taken at the beginning and end of each collection period. Inulin concentrations in plasma and urine were measured by the colorimetric method and the absorbance was read at 550 nm using a Glomax Multi + detection system (Promega). The GFR was calculated as: (perfusion rate × perfusate inulin) / (plasma inulin) where perfusate inulin and plasma inulin were the inulin concentrations in perfusate and plasma, respectively.

### LIPID PEROXIDATION ASSAY

Lipid peroxidation as index of the oxidative stress was determined by assaying the malondialdheyde (MDA) production by means of the thiobarbituric acid (TBA) test by Esterbauer and Cheeseman [1990]. Kidney homogenates were obtained using a tissue homogenizer Tissue Lyser LT (Quiagen) at 4 °C. The homogenates (1:10 w/v) were prepared by using a 100 mM KCl buffer (7:00 pH) containing EDTA 0.3 mM. Briefly: 1 ml of supernatant of renal tissue was mixed with

0.5 mL of cold 30% (w/v) trichloroacetic acid to precipitate proteins. The precipitate was pelletted by ultracentrifugation and 1 ml of the supernatant was reacted with 1.3 ml of 0.5% (w/v) TBA at 85 °C for 40 min. In TBA test reaction, one molecule of MDA reacts with two molecules of TBA with the production of a pink pigment having maximal absorbance at 532–535 nm. Therefore, after cooling, the fluorescence was read at an excitation of 536 nm and an emission of 557 nm wavelengths in a Glomax Multi + detection system spectrophotofluorimeter (Promega). The concentration of MDA was calculated respect to a calibration curve (range: 0.5–2 pmoles/ml), and results were expressed as pmoles of MDA/mg of proteins.

### SOD ACTIVITY ASSAY

The assay for SOD activity involves inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as a superoxide generator [Sun et al., 1988]. SOD activity was determined by the sensitive SOD assay kit that utilizes a product a water-soluble formazan dye upon reduction with superoxide anion SOD activity with absorbance at 450 nm according to the manufacturer's instructions (Assay Kit Caymac Chemical Company). Briefly, 1 ml of supernatant of renal tissue treated or not treated with OTA and OTA in combination with rMnSOD was mixed. At the end of incubation the supernatant was lysed and centrifuged at 14,000 g for 5 min at 48 °C and on cytosol was determined cytosolic and mitochondrial SOD activity. The starter of reaction by adding Xanthine Solution. The absorbance readings every minute for 10 min at room temperature. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. The SOD activity was expressed as units for milligrams of protein.

### CAT ACTIVITY ASSAY

The colorimetric assay has been applied to evaluate catalase (CAT) activity [Sinha, 1972]. The method is based on the principle that dichromate in acetic acid is reduced to chromate acetate when heated in the presence of $H_2O_2$, with the formation of perchromic acid as an unstable intermediate. The chromic acetate thus produced was measured colorimetrically and absorbance was read at 520 nm according to the manufacturer's instructions (Assay Kit Caymac Chemical Company). Results were expressed as units for milligrams of protein.

### RENAL HISTOLOGY

The kidneys were removed and immediately fixed by immersion in Bouin's fixative (6–24 h). The specimens then were processed for paraffin embedding in vacuum and cut at a thickness of 5–7 μm. Paraffin sections were stained by using haematoxylin-eosin in order to study the renal histology. In addition, paraffin sections were stained with Masson's trichrome staining. The pictures were taken at a ×250 magnification (n = 5 section was examined) by using a Leica DMRA2 microscope. The tissues were examined without knowledge of the rat's identity.

### TOTAL PROTEIN ASSAY

Total proteins were determined by the method of Lowry et al. [1951].

### STATISTICAL ANALYSIS

All data are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA followed by the unpaired *t*-test. A value of $P < 0.05$ was considered statistically significant.

## RESULTS

### BLOOD PRESSURE MEASUREMENT

As shown in the Figure 1, OTA significantly increased BP, expressed as mmHg, at 14 days of the treatment compared to control. In fact BP values shifted from 94 ± 6.10 (control) to 134 ± 4.01 mmHg (OTA) ($^{*}P < 0.05$). However, the administration of rMnSOD in combination with OTA restored this function from 134 ± 4.01 (OTA) to 96 ± 2.12 (rMnSOD + OTA) mmHg ($^{\#}P < 0.05$). rMnSOD, utilized alone, did not exert any change.

### CLEARANCE OF INULIN

In the Figure 2 has shown the OTA effect on renal haemodynamics. GFR, expressed as ml/min 100 g bw, was measured by means of inulin clearance. OTA treatment significantly decreased GFR compared to control animals. In fact GFR values shifted by from 0.98 ± 0.02 (control) to 0.63 ± 0.03 (OTA) ml/min ($^{*}P < 0.05$). The treatment with rMnSOD in combination with OTA, exerted a good protection on OTA-induced GFR reduction. In fact the GFR values shifted from 0.63 ± 0.03 (OTA) at 0.89 ± 0.05 (rMnSOD + OTA) ml/min ($^{\#}P < 0.05$). rMnSOD utilized alone, did not exert any change on glomerular function (0.85 ± 0.07 respect to 0.98 ± 0.02 control).

### MDA LEVELS

n the Figure 3 has shown the OTA effects on lipid peroxidation measured by the malondialdheyde (MDA) levels in rat kidney after 2 weeks of treatment. Renal MDA level, expressed as pmoles/mg proteins, increased in OTA group respect to the control. In fact the MDA values shifted from 440 ± 11.21 (control) to 550 ± 9.12 (OTA) pmoles/mg proteins ($^{*}P < 0.05$). When rMnSOD was used in association with OTA, we have observed a restore of this value (550 ± 9.12 (OTA) to 458 ± 10.91 (OTA + rMnSOD)) pmoles/mg

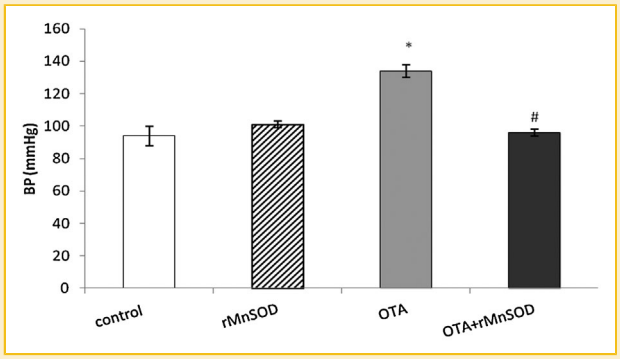


**Fig. 1.** Effects of OTA and rMnSOD used alone or in association on blood pressure (BP) in rats treated for 2 weeks. OTA treatment significantly increased BP, while co-administration with rMnSOD partially restored this effect (values mean ± SD; $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. OTA group).

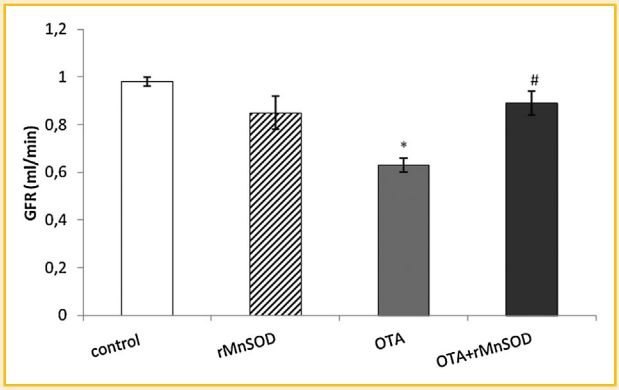


Fig. 2. Effects of OTA and rMnSOD used alone or in association on glomerular filtration rate (GFR) after 2 weeks of treatment. OTA treatment significantly decreased GFR, while co-administration with rMnSOD partially restored this effect (values mean ± SD; $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. OTA group).

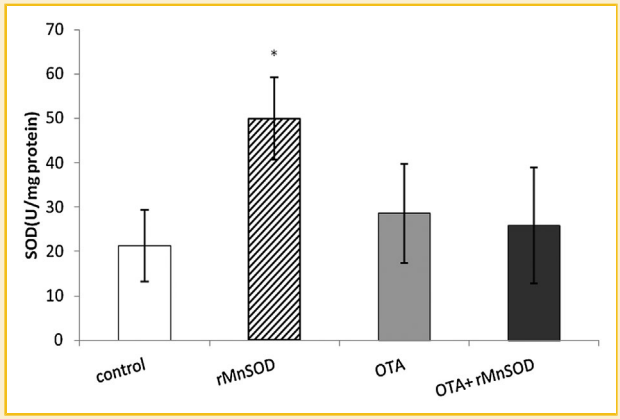


Fig. 4. Effects of OTA and rMnSOD used alone or in association on SOD enzymatic activity in rat kidney after 2 weeks of treatment. OTA treatment did not show significant change in SOD enzymatic activity respect to control. The same result is obtained during co-administration with rMnSOD. rMnSOD used alone showed a significant change in SOD enzymatic activity respect to control (values are mean ± SD; $^{*}P < 0.05$ vs. control group).

proteins ($^{\#}P < 0.05$). rMnSOD utilized alone, did not exert any change on lipid peroxidation (410 ± 10.50 respect to 440 ± 11.21 control).

## SOD AND CAT ACTIVITY

As shown in the Figure 4, OTA and rMnSOD + OTA groups they have not changed total SOD activity compared to control. In fact the value observed were of 21.24 ± 8.11 (control) respect 28.56 ± 9.21 (OTA) and 25.84 ± 11.11 (rMnSOD + OTA) U/mg protein. rMnSOD, alone, caused a significant increase in SOD enzyme activity compared to control group. In fact the SOD values shifted from 21.24 ± 8.11 (control) to 49.94 ± 13.09 (OTA) U/mg proein ($^{*}P < 0.05$). The OTA and OTA + rMnSOD did not exert any change on SOD activity.

Figure 5 shows the OTA effect on CAT activity. After 14 days of treatment with OTA and rMnSOD + OTA group they have not changed the CAT activity compared of control. In fact the value observed were of 356 ± 11.83 (control) respect 320 ± 19.11 (OTA) and 331 ± 19.09 (rMnSOD + OTA) U/mg protein). The treatment with rMnSOD utilized alone showed a significant effect on CAT activity respect to control. In fact the SOD values shifted from 356 ± 11.83 (control group) to 480 ± 21.11 (rMnSOD group) U/mg protein ($^{*}P < 0.05$).

## HISTOPATHOLOGICAL EXAMINATION

The normal histological structure of the kidney was observed in Figure 6. Section of the kidneys of control rats showed normal appearance of glomerulus and renal tubules. On the other hand, kidneys of rats treated with OTA for 14 days showed vacuolar

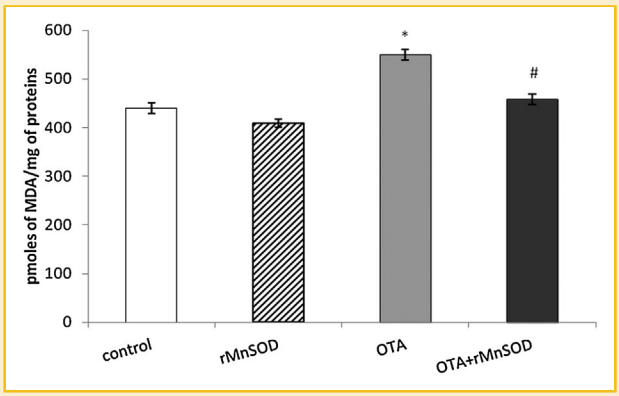


Fig. 3. Effects of OTA and rMnSOD used alone or in association on lipid peroxidation measured by malondialdehyde (MDA) test in rat kidney after 2 weeks of treatment. OTA treatment significantly increased MDA levels, while co-administration with rMnSOD partially prevent this effect (values are mean ± SD; $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. OTA group).

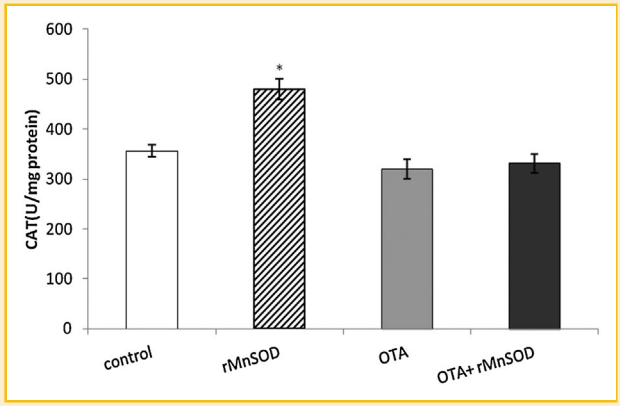


Fig. 5. Effects of OTA and rMnSOD used alone or in association on CAT enzymatic activity in rat kidney after 2 weeks of treatment. OTA treatment did not show significant change in CAT enzymatic activity respect to control. The same result is obtained during co-administration with rMnSOD (values are mean ± SD, $^{*}P < 0.05$ vs. control group).

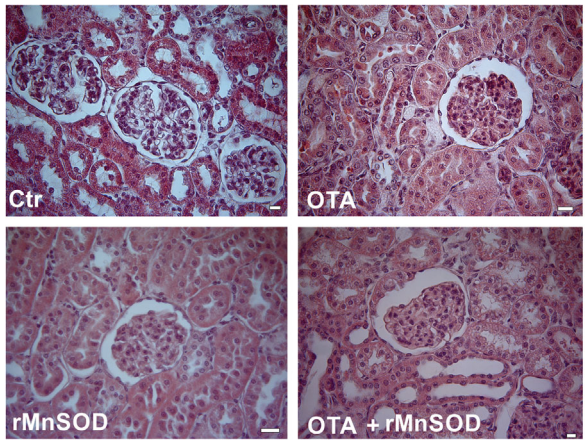


**Fig. 6.** Effects of OTA and rMnSOD used alone or in association on morphological alteration in rat kidney after 2 weeks of treatment. rMnSOD prevents OTA induced morphological alteration. Representative pictures of haematoxylin and eosin staining from the renal cortex. OTA treatment showed glomerular and tubular alteration. Co-treatment with rMnSOD is associated with a partially restored of this histological alteration. Magnification ×250.

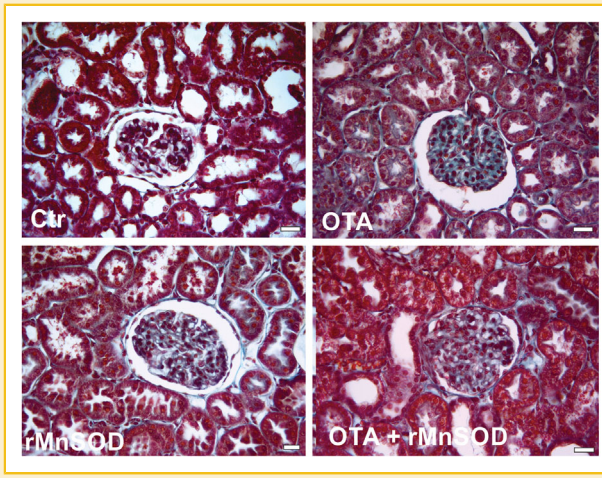


**Fig. 7.** Effects of OTA and rMnSOD used alone or in association on interstitial fibrosis evaluation in rat kidney after 2 weeks of treatment. rMnSOD prevents OTA induced interstitial fibrosis. Representative pictures of Masson's trichrome staining from renal cortex. OTA treatment showed interstitial fibrosis. Co-treatment with rMnSOD is associated with a partially restored of this histological alteration. Magnification ×250.

degeneration of the tubular epithelium, reduction in the height of the tubular epithelium, glomerular degeneration, presence of cell debris in the lumen of the tubule and interstitial infiltrates. These morphological alterations were mainly located in the cortex and outer stripe of outer medulla and they were common both in proximal and distal tubules. The treatment of rats with rMnSOD and OTA in association showed that tubular and glomerular degeneration was decreased. rMnSOD treatment alone was not associated with significant morphological alteration, compared to control group.

In the Figure 7 has shown the OTA effect on interstitial fibrosis evaluated by Masson's trichrome staining. OTA treatment induced a more severe interstitial fibrosis compared to control group. Co-treatment of rMnSOD and OTA was associated with a decrease of fibrosis compared with the OTA group alone. rMnSOD treatment alone was not associated with significant interstitial fibrosis compared to control group.

## DISCUSSION

OTA is involved in several animal and human diseases [Krogh, 1976; Bozić et al., 1995]. In humans, it has been related to Balkan endemic nephropathy and in animals, chronic exposure to OTA, produces a well described nephropathy [Ribelin et al., 1978; Boorman et al., 1992; Grollman et al., 2007; Bui-Klimke and Wu, 2015]. In addiction, chronic studies have shown a clear relationship between OTA administration and the incidence of renal tumor in rat and in mouse [Bendele et al., 1985; O'Brien et al., 2005].

The exact mechanism of OTA toxicity has not been yet characterized. However, several studies have proposed the involvement of reactive oxygen species to the nephrotoxicity induced by OTA [Klaunig et al., 1998; Palabiyik et al., 2013]. In particular, the gene expression profiles have revealed the involvement of the antioxidant regulatory element (ARE) in their promoter region that is recognized by the nuclear factor -erythroid 2 p45-related factor 2 (Nrf2) [Bendele et al., 1985]. Nrf2 involved in the expression and induction of many genes, including genes for detoxification and antioxidant enzymes. Several studies [Hayes et al., 2000; Kensler et al., 2007] have shown that the nephrotoxicity induced by OTA is related to DNA damage induced by oxidative stress rather than a direct interaction of OTA with the cellular DNA.

The present study was designed to investigate the relationship between OTA exposure and ROS production in the development of renal injury in rats treated for 2 weeks with oral injection of OTA and the potential effect of the new recombination rMnSOD in the treatment of OTA nephrotoxicity. We have measured the blood pressure, the GFR alterations, the renal histology damage, the change in lipid peroxidation, catalase and superoxide dismutase activities to clarify the role of oxidative stress in the OTA nephropathy.

Our data, the first reported so far, show that OTA induced hypertension in treated rats. There are no data in the literature explaining this effect, but it could be argued that this effect could be to an increase of ROS productions. In fact, it has been demonstrated in various experimental models of hypertension, that the increase of blood pressure is linked to ROS increase and it is prevented by the treatment with antioxidants [Gill and Wilcox, 2006; Palabiyik et al., 2013]. In an our previous article we have shown that the use of antioxidant apocynin in CsA hypertensive rats restored blood pressure and levels of ROS [Ciarcia et al., 2015]. Here we have shown that treatment with the new recombinant rMnSOD prevents hypertension induced by OTA and, in addiction, restores lipid peroxidation levels. Therefore, we could hypothesize that the mechanism responsible of this result is linked to the rMnSOD ability to scavenge most of the radical species. This property is probably

related to the presence of a leader peptide that allows rMnSOD to enter the cell thus preventing its degradation by circulating proteases [Mancini et al., 2006], but further studies are needed.

It has been previously demonstrated that OTA induces also a severe renal functional disorder with an abnormal creatinine and BUN levels [Palabiyik et al., 2013]. Indeed, in our experiments, we have found a severe decrease in the GFR measured by inulin clearance, which is considered the gold standard method for the measurement of renal function. Gekle and Silbernagl [1993] have previously demonstrated in OTA treated rats that the decrease in GFR was mediated by Angiotensin II (ANGII) increase. It is well demonstrated that the increase in ANG II is accompanied by oxidative stress and an increase in $O_2^-$ generation [Gill and Wilcox, 2006] reducing the bioavailability of the vasodilator nitric oxidase and inducing a severe glomerular vasoconstriction with, consequently, a reduction in GFR. Here we have shown that rMnSOD restored the GFR. Therefore, although the mechanism responsible for the GFR reduction in OTA hypertensive rats has not been well defined, we hypothesized that the reduction of GFR is related to the increase of ANG II and levels of $O_2^-$. It is possible that the mechanism responsible for these results on GFR is linked to the ability of rMnSOD to scavenge most of the radical species, since it was demonstrated that nephrotoxicity induced by OTA is linked to ROS production and to an imbalance between oxidant/antioxidant parameters [Schaaf et al., 2002].

Our data evidenced a severe histological damage as results of OTA treatment. In fact, we have observed that OTA induced alteration on tubular epithelium, glomerular degeneration and tubule and interstitial infiltrates. Moreover, interstitial fibrosis has been observed. In agreement with our results, Palabiyik et al. [2013] showed a tubular degenerative change such as epithelial cell desquamation and detachment in OTA treated rats. Moreover, Abdu et al. [2011] have found a global congestion in the renal tissue, loss of demarcation between the cortex and medulla and pre-apoptotic cells in rats treated for 28 days with OTA. Therefore, the activation of damage and necrosis by OTA could be the cause of the kidney damage and kidney failure.

Several mechanisms of OTA nephrotoxicity have been proposed, such as the inhibition of protein synthesis, promotion of lipid peroxidation, inhibition of mitochondrial respiration and DNA damage [Bendele et al., 1985; Hayes et al., 2000; O'Brien et al., 2005; Kensler et al., 2007].

Our data, according to the result of Palabiyik et al. [2013], demonstrated that OTA did not modify SOD and CAT enzymatic activity in treated rats. rMnSOD used alone increased both CAT and SOD levels but did not modify such activity when used in association with OTA. Thus, it could be hypothesized that the restoring potential of rMnSOD on OTA nephrotoxicity is related to its direct scavenging in activity on ROS, rather than to its modulation of cellular enzymatic activities.

In this work we have showed that the hypertensive effect OTA, the decrease in GFR and renal histological damage is due to the increase of ROS production. In addition, rMnSOD is able to prevent in vivo the deleterious effects of OTA and confirmed its potential therapeutic role in the treatment of OTA nephrotoxicity.

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