

Original Article

***In vivo* effect of the natural antioxidant hydroxytyrosol on cyclosporine nephrotoxicity in rats**

Giovambattista Capasso¹, Chiara Iolanda Di Gennaro², Fulvio Della Ragione², Caterina Manna², Roberto Ciarcia³, Salvatore Florio³, Angelica Perna¹, Rosa Maria Pollastro¹, Sara Damiano¹, Orazio Mazzoni⁴, Patrizia Galletti² and Vincenzo Zappia²

¹Chair of Nephrology, Department of Internal Medicine, ²Department of Biochemistry, Second University of Napoli,

³Department of Biological Structure and Function and ⁴Department of Pharmaceutical and Toxicological Chemistry, University of Naples Federico II, Napoli, Italy

Abstract

Background. Cyclosporine A (CsA) is the first-line immunosuppressant used in transplant patients and in autoimmune diseases. Nephrotoxicity is the major limitation of CsA use. Although the mechanisms of nephrotoxicity have not been completely defined, some evidence suggests that reactive oxygen species (ROS) play a causal role. The present study was designed to investigate *in vivo* effects of hydroxytyrosol (DOPET), a natural olive oil antioxidant, on oxidative stress, renal histology and haemodynamic alterations induced in rats by CsA treatment.

Methods. Adult Sprague–Dawley rats were treated i.p. with CsA (15 mg/kg) alone or in combination with DOPET (20 mg/kg) for 3 weeks. At the end of the treatment, superoxide concentration within the cells of the abdominal aorta and renal artery was quantified from the oxidation of dihydroethidium (DHE) using fluorescence microscopic imaging analysis. In kidney tissues, lipid peroxidation was measured by thiobarbituric acid-reacting substances (TBARS) assay, glutathione level was assessed enzymatically and the expression of haem oxygenase-1 (HO-1) gene was evaluated by semiquantitative RT-PCR. Renal morphology was studied by classical histological techniques, while the glomerular filtration rate (GFR) was estimated by inulin clearance. Systemic blood pressure was monitored by the tail method and through the catheterization of the carotid artery.

Results. CsA administration increased superoxide concentration both in the aorta and in the renal artery, while DOPET completely prevented this effect. Higher levels of TBARS, a significant decrease in GSH and an upregulation of HO-1 mRNA were observed in the kidneys of CsA-treated rats. DOPET treatment reversed quantitatively these effects. However, CsA-dependent changes in renal

histology were only partially reversed by DOPET. Finally, CsA induced a severe reduction in GFR and a significant increase in both systolic and diastolic blood pressure; the DOPET treatment had no significant effect on these haemodynamic alterations.

Conclusion. The reported data indicate that effective DOPET protection from CsA-induced oxidative stress is associated with a mild effect on histological damages and does not affect the altered glomerular function and the hypertension, thus indicating that kidney injury by CsA is only in part dependent on oxidative stress.

Keywords: blood pressure; cyclosporine A; free radicals; hydroxytyrosol; nephrotoxicity

Introduction

Cyclosporin A (CsA) is widely utilized as the immunosuppressant of choice in organ transplantation, as well as in the therapy of several immunodisorders [1]. However, its clinical use is often limited by the frequent and dose-dependent severe side effects. Nephrotoxicity is the main untoward consequence of CsA treatment. It has been shown that after a decade of CsA administration, all the patients had evidence of nephrotoxicity [2]. On the basis of the length of treatment, two forms of CsA nephrotoxicity have been described: acute and chronic. Acute CsA treatment induces reversible reduction of the glomerular filtration rate (GFR) and renal blood flow that is related to afferent arteriolar vasoconstriction [3]. This may be related to an increase in vasoconstrictors factors such as endothelin [4], thromboxane [5], angiotensin II [6] and/or a decrease in vasodilators factors such as prostacyclin [7] and nitric oxide (NO) [8]. Thus an imbalance in the release of vasoactive substances may account for renal vasoconstriction. When given chronically, CsA can lead to irreversible renal failure due to renal vasoconstriction, but also

Correspondence and offprint requests to: Giovambattista Capasso, Chair of Nephrology, Second University of Napoli, Padiglione 17 Policlinico Nuovo, Via Pansini 5, 80131 Napoli, Italy. Tel: +39-081-5666652; Fax: +39-081-5666652; E-mail: gb.capasso@unina2.it

to tubulointerstitial fibrosis, tubular atrophy and glomerular sclerosis [2]. Several studies have been undertaken to investigate the mechanisms underlying these unwanted side effects. These are: (1) activation of the renin–angiotensin–aldosterone system [9]; (2) upregulation of transforming growth factor- β (TGF- β) [10]; (3) renal hypoxia resulting from renal vasoconstriction [11]. With respect to this last mechanism it has been proposed that the cellular mediators may be the formation of reactive oxygen species (ROS).

Indeed much experimental evidence has been accumulating on the involvement of oxidative stress in the toxic effects of CsA treatment. *In vivo*, CsA increases lipoperoxidation in the rat kidney and liver, depletes the hepatic and renal pool of glutathione [12] and impairs the antioxidant defence system. Moreover, it has been reported that the mRNA and protein levels of haem oxygenase-1 (HO-1), an enzyme responsive to changes in the redox status, vary after treatment with CsA [13,14].

Several hypotheses have been proposed to explain the link between CsA treatment and ROS: (1) upregulation of kidney cytochrome P-450-dependent ROS producing system [15]; (2) perturbation of the vasoconstriction–vasodilation balance, in turn responsible for tubular hypoxia-reoxygenation [16]; (3) increased formation of renal thromboxane A2 [17]; (4) induction of NO production [18].

The hypothesis that CsA toxicity is mediated by ROS led investigators to use antioxidants to prevent or ameliorate its toxicity [19]. In cultured hepatocytes, vitamin E totally prevents CsA cytotoxicity as well as lipid peroxidation, and increases the expression and activity of several endogenous antioxidant enzymes. This vitamin also preserves the renal function and structure when administered *in vivo* to CsA-treated rats [20].

However, the view that antioxidants might exert protective effects against CsA adverse actions is called into question by our recent findings. In a previous *in vitro* study investigating the possible protective effect of natural phenolic antioxidants against CsA toxicity in rat tubular cells [21], we demonstrated that hydroxytyrosol (DOPET, 3,4-dihydroxyphenylethanol) although able to prevent the CsA-induced production of ROS and to counteract the increase in lipoperoxidation, failed to exert any protection against CsA cytotoxicity. In contrast, vitamin E confirmed its protective effect also in this model system. These unexpected results indicate that ROS-independent pathways may be the primary cause of CsA-mediated cytotoxicity and that vitamin E protection is probably related to non-antioxidant properties of this molecule [21]. Accordingly, in the past few years, several roles have been attributed to vitamin E besides its chain breaking activity, including modulation of gene expression and cellular signalling [22].

To further elucidate whether ROS formation is responsible for CsA toxic effects, in the present study we investigate the possible protective role of DOPET on *in vivo* nephrotoxicity induced in rats by chronic CsA treatment. This antioxidant, which naturally occurs in extra-virgin olive oil contributing to its health beneficial properties, is characterized by low toxicity and is mainly metabolized and excreted by the kidney when intravenously injected [23]. Moreover, DOPET, a powerful scavenger of peroxyl radicals, prevents

in vitro LDL oxidation, platelet aggregation and inhibits 5- and 12-lipoxygenases [24].

Materials and methods

Experimental protocol

Experiments were performed on a total of 64 Sprague–Dawley rats, weighing 200–230 g, housed under constant environmental conditions (temperature 22°C and a 12-h light–dark cycle). Animals were fed a standard stock chow diet; food and water were given *ad libitum*. The rats, randomly divided into four groups, were treated for 3 weeks as follows: (a) group 1 was given a daily i.p. injection of CsA vehicle (Cremophore EL and alcohol in a 2:1 ratio, in saline solution); (b) group 2 was treated i.p. with 15 mg/kg/day CsA; (c) group 3 was i.p. treated with both 15 mg/kg/day CsA and 20 mg/kg/twice daily DOPET; (d) group 4 was treated with 20 mg/kg/twice daily DOPET alone. Diastolic blood pressure (DBP) and systolic blood pressure (SBP) were measured on conscious rats using the tail method. Repeated measurements were taken on Days 0–5–10–15–20 using a blood pressure recorder (LE 5002 Storage Pressure Meter, Ugo Basile, Italy). The animals were adapted to the BP recording for at least 4 days before starting the experiments. BP was also recorded in anaesthetized rats, during the clearance experiments, through the carotid artery using a blood pressure recorder. The dose and length of CsA administration was chosen since nowadays, it is normally accepted that 15 mg/kg/day CsA induces acute and chronic nephrotoxicity on the basis of the time treatment. The dose of DOPET was chosen on the basis of the previous *in vitro* study [21].

Superoxide assay in the dissected aorta and renal artery

Superoxide concentrations within cells of the abdominal aorta and renal artery were monitored by measuring the changes in fluorescence resulting from the oxidation of dihydroethidium (DHE; Molecular Probes). DHE can enter the cell and be oxidized by superoxide to yield ethidium (Eth), which binds DNA producing bright red fluorescence. The increase in Eth-DNA fluorescence indicates peroxide production within cells [25]. At the time of the sacrifice, arteries were identified, frozen at -80°C and dissected with a cryostat (Leica) into 5- μm -thick slices, and then incubated with a 20 μM DHE solution for 30 min at room temperature. At the end of the incubation time, the slices were mounted on the stage of a fluorescence microscope (Axiophot 2000) using a 60 nm wavelength, and the fluorescence images were captured with a digital charge-couple device camera controlled by image-analysis software (Adobe Photoshop). The fluorescence intensity was integrated and normalized for the surface area; it is expressed in fluorescence arbitrary units (FAU).

Biochemical experiments

Renal tissue preparation At the end of the *in vivo* treatment, the rats were killed by exsanguination and the right

kidneys were quickly removed and washed with ice-cold isotonic saline solution. The kidneys were homogenized in 1.15% NaCl, containing 5% SDS, 1:3 (w/v). The homogenate was centrifuged at 10 000 g for 1 h at 4°C and the supernatant was utilized for biochemical assays.

GSH and GSSG assay Glutathione was measured by the glutathione reductase/5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) recirculating assay. The supernatant (1 ml) was treated with sulphosalicylic acid (10% final concentration) and centrifuged at 13 000 g for 15 min. To measure the total glutathione (i.e. GSSG + GSH), 50 µl of sample were incubated in the presence of 150 µl DTNB, 50 mM potassium phosphate buffer (pH = 7.0), 200 µM NADPH, 1 mM EDTA in 1 ml final volume. The reaction was initiated by addition of 1.7 IU GSSG-reductase. After 10 min incubation at room temperature the absorbance was measured at 412 nm. To selectively measure the tissue GSSG content, 200 µl of supernatant were immediately mixed with 2-vinylpyridine (0.6% final concentration) to prevent artefactual oxidation of GSH. The samples were left for 1 h at room temperature and treated as previously described. Glutathione concentrations were expressed as nanomoles of GSH/g of kidney wet weight. GSH concentration was calculated from the difference between total and oxidized glutathione.

TBARS assay Lipoperoxidation was evaluated by the TBARS (thiobarbituric acid reactive substances) assay modified as follows: 0.4 ml of supernatant was treated with 1 ml of 20% acetic acid and 1 ml of 1% TBA and incubated for 15 min at 90°C in a water bath. After cooling, the samples were extracted with 2 ml of butanol/pyridine (15:1) and the absorbance measured at 532 nm. TBARS concentration, expressed as nmol/g kidney wet weight, was obtained using the value of 153 000 mol/l/cm as the extinction coefficient of TBA adducts.

Analysis of HO-1 gene expression by semiquantitative RT-PCR

The left kidney was isolated immediately after animal sacrifice, and the renal cortex, outer medulla and inner medulla were dissected at 4°C. These biological samples were transferred into individual tubes containing 1 ml TRIzol reagent (Life Technologies, Gaithersburg, MD) and then incubated at room temperature for 5 min.

Total RNA was extracted and purified as previously described [26]. The primers and the PCR conditions employed for the various genes are reported below. All the reactions had a hot start of 10 min at 95°C and a final elongation step of 7 min at 72°C. GAPDH: 5'-CAATGTATCCGTTG-TGGATCTGACAT-3' (sense) and 5'-ATATTCATTGTCA-TACCAGGAA ATGAGC-3' (antisense); 20 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min. HO-1: 5'-TGGAAGAG GAGATAGAGCGA-3' (sense) and 5'-TGTTGAGCAGGAA GGCGGTC-3' (antisense); 33 cycles of steps at 95°C for 1 min, 58°C for 1 min and 72°C for 1 min.

Before amplifications with each specific primer pair, an aliquot of the cDNA preparation was amplified using

GAPDH primers to determine the cDNA structural integrity. Moreover, we used five different cDNA concentrations to ensure that the signals (both of GAPDH and HO-1) were proportional to the mRNA input. Each experiment was performed at least in triplicate and, in several cases, in quadruplicate.

Whole kidney clearance

On 16 animals, treated as described above, GFR was measured at the end of the experimental treatment. The rats were anaesthetized with an intra-peritoneal injection of Inactin (Sigma-Aldrich, St. Louis, MO, USA), 120 mg kg⁻¹ body wt, tracheostomized, placed on a thermo-regulated table (37°C) and prepared for renal clearance evaluation. In brief, the right carotid artery was catheterized to monitor blood pressure through a blood pressure recorder (BP1 by WPI, USA) 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 inulin from 10% saline solution. The surgical procedure also included bladder catheterization with PE-50 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 start and end of each collection period. Inulin concentrations in plasma and urine were measured by the colorimetric method. GFR was calculated using standard clearance formulae.

Morphology

Using 28 animals, treated as above, morphological data were collected. To this end, the kidneys were removed, cut sagittally and fixed with 4% buffered paraformaldehyde at room temperature. Tissues were dehydrated in gradual alcohols and embedded in paraffin. Four to five thick sections were stained with periodic acid-Schiff's reagent (PAS) and Trichrome. The histological findings were subdivided into three groups: tubular injury, interstitial inflammation and fibrosis, and arteriopathy. A minimum of 10 fields at ×100 and ×400 magnification were assessed and graded in each biopsy. The tissues were examined without knowledge of the rat's identity.

Semiquantitative score was used to assess the extent of changes in each group [27]. For tubular injury the following score was used: 0 = no tubular injury; 1 = <25% of tubules injured; 2 = from 25% to 50% of tubules injured; 3 = from 51% to 75% of tubules injured; 4 = more than 76% of tubules injured. Interstitial damage was estimated semiquantitatively using the following scores: 0 = normal interstitium; 1 = up to 25% of areas injured; 2 = from 26% to 50% of areas injured; 3 = from 51% to 75% of areas injured; 4 = more than 76% of areas injured. The hyalinosis, identified through the PSA staining, consisted of hyaline deposition within the tunica media of afferent arterioles and terminal portions of interlobular arteries. It was assessed on the afferent arterioles using the scores as follows: 0 = no arterioles injured; 1 = up to 25% of arterioles injured; 2 = from 26% to 50% of arterioles injured; 3 = from 51% to 75% of arterioles injured; 4 = >76% of arterioles injured.

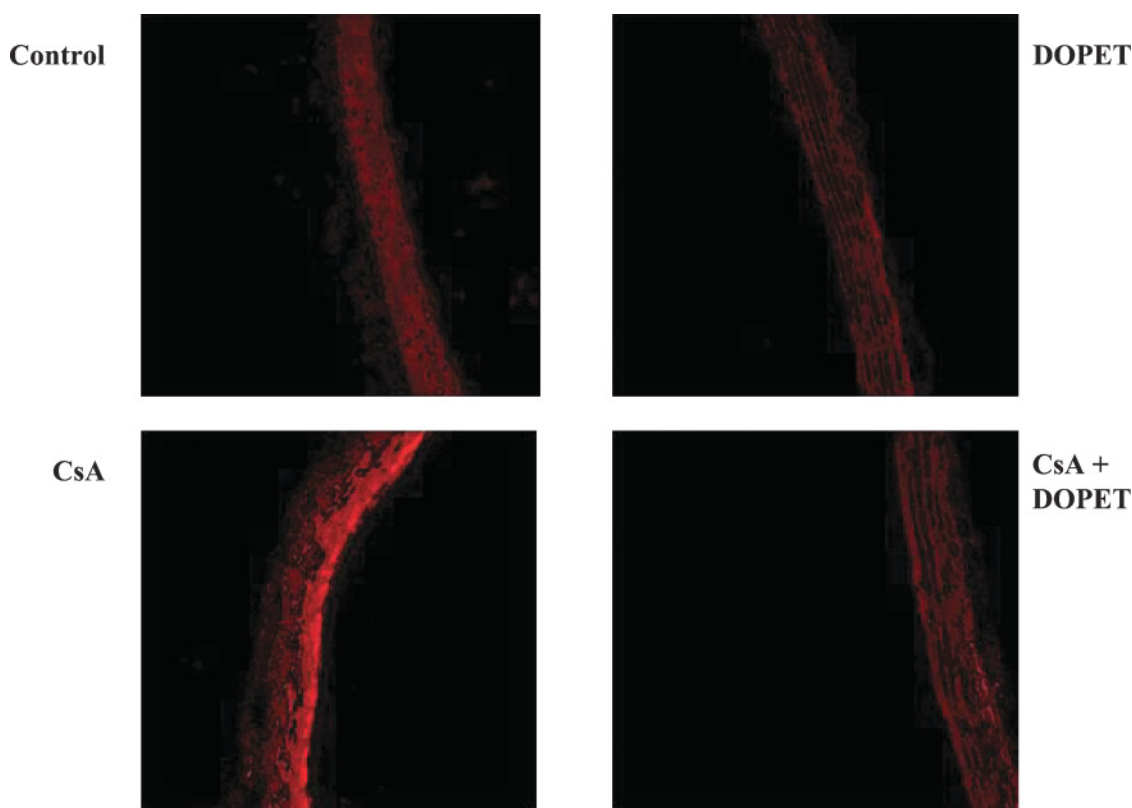


Fig. 1. Effect of CsA (15 mg/kg/day) and DOPET (20 mg/kg/twice daily) on the production of superoxide in the abdominal aorta. Superoxide concentrations within cells of the abdominal aorta were monitored by measuring the changes in fluorescence resulting from the oxidation of dihydroethidium (DHE). It seems that CsA induces an increase in the thickness of tunica media, a lesion that is also apparent from the histological data.

Reagents

CsA as Sandimmune (intravenous preparation), containing Cremophore EL and alcohol as vehicle (2:1) was provided by Novartis (Basel, Switzerland). DOPET was synthesized according to Baraldi with some modifications [28]. All other chemicals were purchased from Sigma-Aldrich.

Statistical analysis

All the data are expressed as mean \pm SEM. Statistical analysis was performed by one-way ANOVA followed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

Rats were treated with humane care, in compliance with the indications of the *Guide to the Care and Use of Experimental Animals*.

Results

Effects of DOPET on CsA-induced oxidative stress

The red fluorescence generated by the binding of ethidium-DNA complex is considered an appropriate indicator of superoxide production within the cells as described in the Materials and Methods section. Using this procedure, we show that the abdominal aorta of CsA-treated animals presents a red fluorescent signal significantly brighter than control (Figures 1 and 2, panel A). When the rats were treated with

CsA plus DOPET the fluorescence intensity was similar to controls, indicating that DOPET was able to completely prevent the production of superoxide induced by CsA (Figures 1 and 2, panel A). Analysis of samples from rats receiving only DOPET treatment indicates that the antioxidant administration had no effect. The same protective effect of DOPET is observable also in the renal artery (Figure 2, panel B).

To evaluate the CsA-induced oxidative alteration of cellular components the level of lipid peroxidation products (TBARS) in the rat kidney was measured. As shown in Table 1, a significant increase ($P < 0.01$) of TBARS is observable in the rat kidney after 3 weeks of CsA treatment. The simultaneous administration of DOPET and CSA reduced TBARS to the control level, thus demonstrating that DOPET was able to completely reverse the effect of CsA on lipid peroxidation. TBARS levels were not altered in the DOPET-treated group compared with the vehicle-treated group.

Because of the pivotal role played by glutathione (GSH) in cellular protection against free radical damages, we evaluated the increase of oxidized glutathione (GSSG) and the decrease of the GSH/GSSG ratio as sensitive markers of oxidative stress [12]. As shown in Table 1, chronic treatment with CsA significantly increased GSSG levels ($P < 0.01$ versus control). The co-administration of CsA and DOPET completely reversed this effect. Accordingly, CsA treatment lowered the cellular [GSH]/[GSSG] ratio by 44% versus

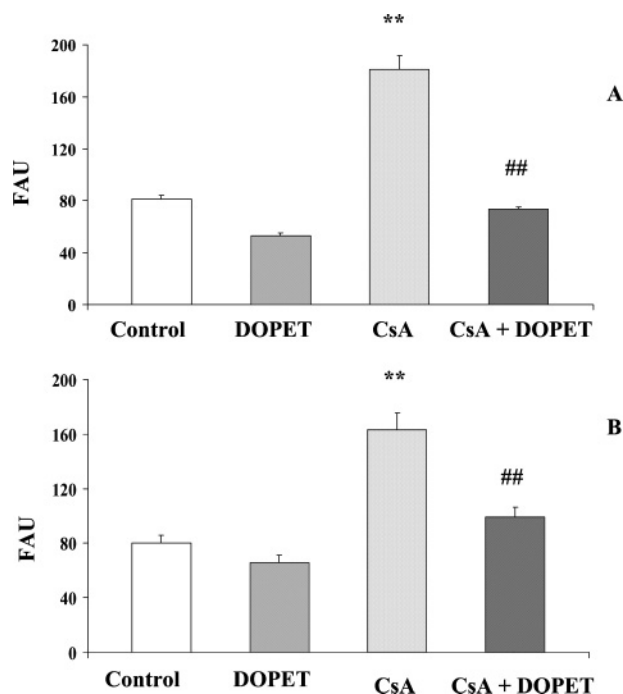


Fig. 2. Effect of CsA (15 mg/kg/day) and DOPET (20 mg/kg/twice daily) on superoxide production in the abdominal aorta (A) and in the renal artery (B). The fluorescence intensity was integrated and normalized for the surface area; it is expressed as fluorescence arbitrary unit (FAU).

Table 1. Oxidative stress markers in CsA treatment

Kidney	TBARS nmol/g tissue	GSSG nmol GSH/g tissue
Control	20.8 ± 3.4	1.66 ± 0.11
DOPET (20 mg/kg)	23.8 ± 2.5	1.50 ± 0.10
CsA (15 mg/kg)	37.2 ± 7.4 ^{a**}	2.17 ± 0.29 ^{a**}
CsA (15 mg/kg) + DOPET (20 mg/kg)	28.0 ± 2.3 ^{b*}	1.60 ± 0.19 ^{b*}

Lipid peroxidation was measured in kidney tissues as thiobarbituric acid-reacting substances (TBARS); oxidized glutathione was assessed by enzymatic assay. Control group received CsA vehicle, CsA group received CsA 15 mg/kg/day and DOPET group received DOPET 20 mg/kg/twice daily. Significance (*t*-test)/ANOVA: ^a = versus control; ^b = versus CsA; **P* < 0.05; ***P* < 0.01.

control, while DOPET completely prevented this alteration (Figure 3).

A number of facts demonstrate that HO-1 gene expression varies under a wide range of conditions, including alterations of the cellular redox state. As shown in Figure 4, CsA treatment resulted in a significant increase of HO-1 transcript in the cortex, outer and inner medulla (*P* < 0.01 versus control), while the administration of DOPET alone did not cause any significant effect. Moreover, the CsA induction of HO-1 gene expression was largely reversed by DOPET, regardless of the investigated section of the kidney (cortex, outer and inner medulla).

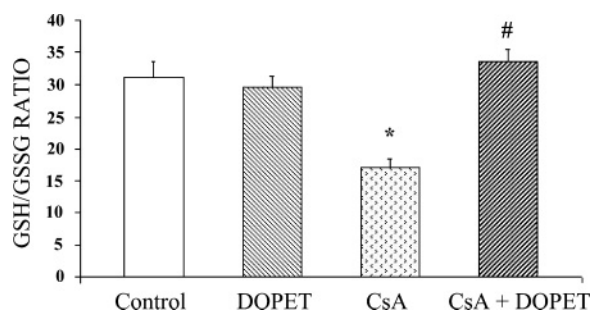


Fig. 3. Effect of CsA (15 mg/kg/day) and DOPET (20 mg/kg/twice daily) on glutathione level expressed as the GSH/GSSG ratio on kidney tissues. ***P* < 0.01 versus control; #*P* < 0.05 versus CsA.

Histological data

Figure 5A and B and Table 2 show respectively the histological lesions characteristic of the four groups of animals and the score obtained according to the procedure outlined in the Method section [27]. DOPET *per se* did not alter the morphology of the renal tissue except sporadic tubular vacuolization revealed by the borderline significance (*P* < 0.05) of the tubular injury score. Conversely, CsA treatment exerted a profound effect on renal histology, at a tubular level, resulting into cellular vacuolization, tubular swelling, dilatation, necrosis and atrophy. The effect on interstitium was exemplified by stripped interstitial fibrosis with the area of sporadic cell infiltrates. The vascular lesion was mainly represented by arteriolar hyalinosis consisting of hyaline deposition within the afferent arteriole wall causing thickening of the media (Figure 5B). The association of DOPET with CsA had only a mild effect on renal damage, as evident from data reported in Table 2 showing that the histological lesions were only partially repaired as compared to control. On the other hand, the vascular lesions were not affected by the DOPET treatment, as evidenced by the lack of a significant difference with the CsA group (Table 2 and Figure 5B).

DOPET effects on CsA-induced hypertension and reduced GFR

As shown in Figure 6, CsA increased both SBP (panel A) and DBP (panel B) by 16–17 mmHg at the end of the treatment period, thus confirming previous data from the literature [30]. However, the administration of DOPET on CsA treated animals did not yield any observable effect on CsA-induced hypertension. DOPET alone failed to cause significant change in blood pressure. As quoted in the Method section, BP was taken through the carotid artery during the clearance experiments. The results demonstrated that compared to controls (mean BP 116 ± 4.3 mmHg, *n* = 5), CsA-treated animals show increased BP that was unaffected by DOPET treatment (CsA + DOPET, 131 ± 3.4 mmHg, *n* = 4, *P* < 0.05 versus control rats), thus confirming the data obtained by the tail method.

In order to evaluate the CsA effect on renal haemodynamics, GFR was measured by means of inulin clearance. The effect of DOPET on chronic CsA-induced renal failure is shown in Figure 7. DOPET *per se* did not exert any

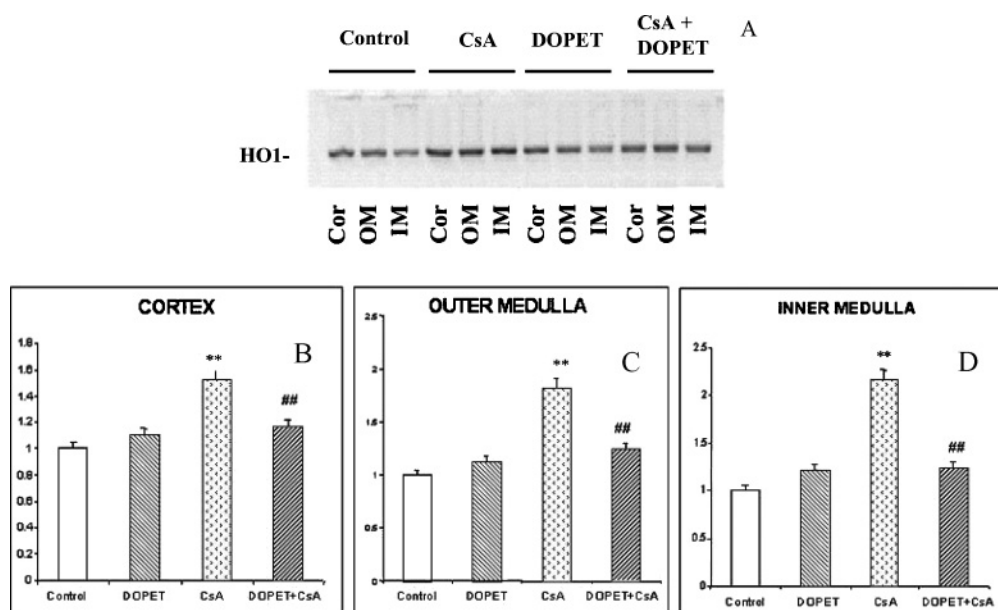


Fig. 4. Effect of CsA (15 mg/kg/day) and DOPET (20 mg/kg/twice daily) on haem oxygenase-1 (HO-1) gene expression. Panel (A) shows the results of the HO-1 gene expression measured by semiquantitative RT-PCR analysis. Panel B reports the results on HO-1 gene transcription in the treated animals expressed as fold of increased with respect of control animals. Cor, cortex; OM, outer medulla; IM, inner medulla. ** $P < 0.01$ versus control; ## $P < 0.01$ versus CsA.

change on glomerular function (0.93 ± 0.05 ml/min·100 g bw). CsA treatment significantly decreased GFR compared to control animals (0.51 ± 0.03 versus 0.94 ± 0.05 ml/min·100 g bw, respectively) ($P < 0.01$), while DOPET, in combination with CsA, did not exert any protection on CsA-induced GFR reduction (0.46 ± 0.02 ml/min·100 g bw).

Discussion

Several researchers have suggested that oxidative stress could play an important role in CsA-induced toxicity [17]. In the present study, in agreement with previous investigations, we demonstrate that chronic treatment with CsA significantly enhances ROS formation in the kidney as revealed by the increased lipid peroxidation (TBARS) and decreased GSH/GSSG ratio. This effect is also confirmed by the significant increase in HO-1 gene expression after CsA treatment. Although this finding does not necessarily correspond to an increase of protein (the mRNA and protein levels not being directly correlated), it is interesting to note that HO-1 is upregulated under a wide range of conditions, all characterized by alterations of the cellular redox state [31]. Furthermore, the production of ROS, following CsA administration, also occurs in other tissues, as demonstrated by directly measuring ROS formation in the aorta and renal artery (DHE experiments).

Among several available antioxidants, the naturally occurring phenol DOPET was chosen to verify the possibility of reducing CsA toxicity *in vivo*. This compound, present in high concentrations in extra virgin olive oil, was selected on the basis of its high scavenging power, elevated bioavailability and *in vivo* low toxicity [32]. Our data show that DOPET completely counteracts CsA-induced oxidative stress as indicated by the increased GSH/GSSG ratio, the

decreased lipid peroxidation level and HO-1 gene expression. Moreover, DHE experiments confirm that DOPET is able to quench CsA-induced ROS production in aorta and renal artery cells. The molecular mechanism of DOPET is directly related to its remarkable ability to scavenge most of the radical species. The present results are in agreement with our previous report indicating that, *in vitro*, DOPET was able to completely prevent CsA-induced ROS generation in rat proximal tubular cells [21].

It has also been reported that CsA administration induces considerable changes in renal morphology; the lesions are mainly tubular, interstitial and arteriolar. In the present experiments we confirm the major characteristics of the CsA-associated renal damages (Figure 5A and B). The co-administration of DOPET with CsA partially affected the documented lesions, with the exception of the damage on the afferent arteriolar that remained unmodified.

Clinical observations [33] and experimental evidence [30] indicate that CsA administration is associated with major side effects including hypertension and renal failure. In our experiments, starting from the second week of treatment, there was an increase of about 15 mmHg in both SBP and DBP values (an observation confirmed also by the measurements through the carotid artery). The mechanism(s) by which CsA causes hypertension are still unclear. Fibrosis and vasoconstriction seem to play a pivotal role in this pathophysiologic process. As stated in the introduction, an imbalance between vasoconstrictors and vasodilators has also been suggested [34]. Among vasodilating compounds, a decrease in circulating NO has been proved to be particularly relevant in producing vasoconstriction, and hence hypertension. It has been proposed that the overproduction of free radicals induced by CsA may lead to the inhibition of NO synthesis with the consequent appearance of hypertension [35].

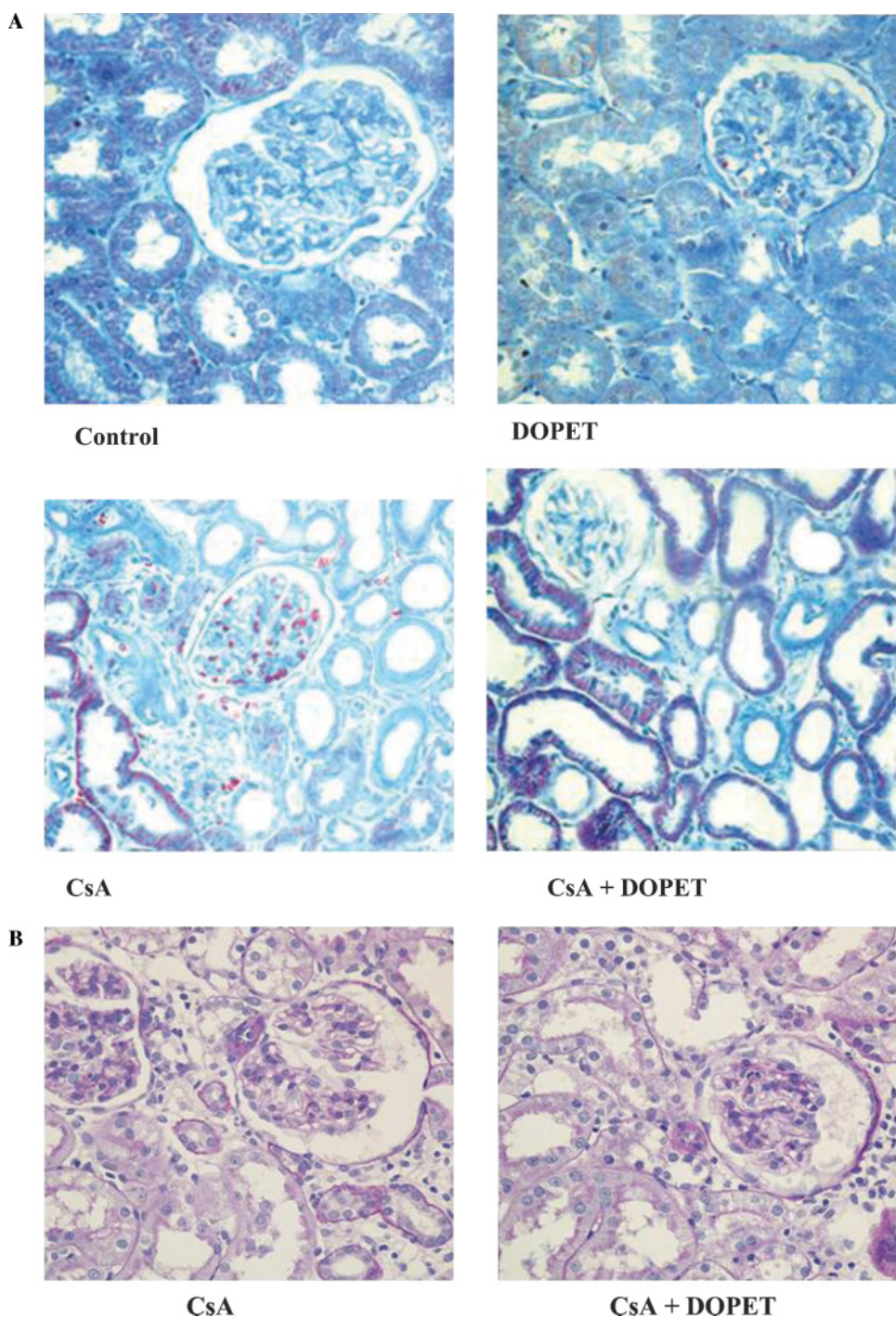


Fig. 5. (A) Effect of CsA (15 mg/kg/day) and DOPET (20 mg/kg/twice daily) on renal histology. DOPET produced a minor change on renal morphology, while CsA-induced profound effect on renal histology characterized by tubular damage and stripped interstitial fibrosis. The use of DOPET associated with CsA had some effect on tubular injury and interstitial fibrosis. In the preparation there are few cells that are blood cells that have not been washed with saline solution. (B) Micrograph on light microscopy of the CsA treated animals. It is shown hyalinosis of the afferent arteriole that remained virtually unchanged in the CsA \pm DOPET group (PAS stain, magnification $\times 400$).

Another side effect of chronic administration of CsA is a severe impairment of renal haemodynamics. In fact, after 3 weeks of CsA administration, a 50% decrease in GFR was observable (Figure 7). Several mechanisms seem to be implicated; however, there is general agreement that the major factor of the CsA effect on GFR is mediated by its action

on afferent arteriolar resistance through vasoconstriction of all contractile elements leading to vessel narrowing [36].

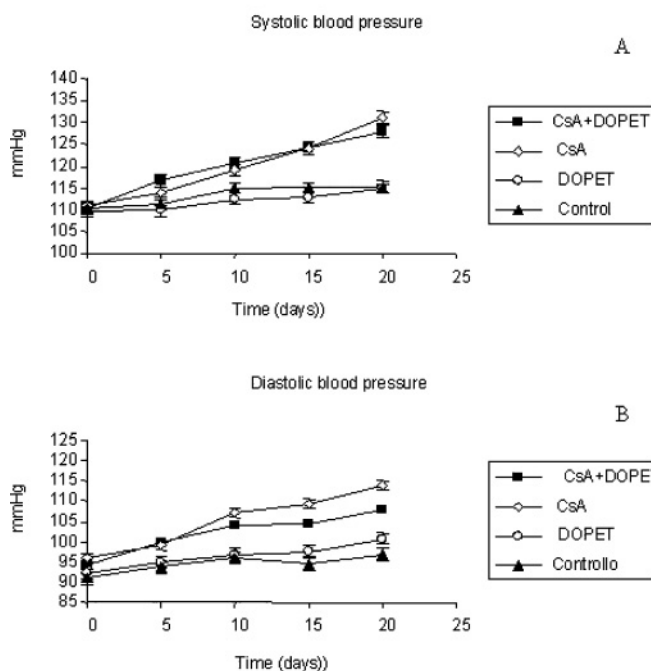
In contrast with its ROS-quenching effect, DOPET was unable to prevent either the increase of blood pressure or the decrease of GFR induced by CsA at least in the present model. It is worth underlining that DOPET alone had almost

Table 2. The renal damage score in the various groups of animals

	Control	DOPET	CSA	CSA + DOPET
Tubular injury	0.014 ± 0.003	0.280 ± 0.100 ^{a*}	1.14 ± 0.09 ^{a***}	0.85 ± 0.07 ^{a***b*}
Tubulointerstitial fibrosis	0.014 ± 0.003	0.011 ± 0.004	1.11 ± 0.23 ^{a***}	0.43 ± 0.20 ^{a*}
Arteriopathy	0.014 ± 0.003	0.014 ± 0.004	0.60 ± 0.13 ^{a***}	0.46 ± 0.19 ^{a*}

Data are mean ± SEM of seven rats for each group. ^aSignificantly different from control; ^bSignificantly different from CSA; **P* < 0.05; ****P* < 0.0001.

BLOOD PRESSURE



Systolic Blood Pressure			
Days	Control	CsA	CsA+DOPET
0	110.3 ± 1.1	110.8 ± 1.4	110.2 ± 2.2
5	111.3 ± 1.5	113.8 ± 1.2	116.7 ± 0.8†
10	115.0 ± 1.7	119.0 ± 1.2††	120.7 ± 1.3†††
15	115.0 ± 1.3	124.0 ± 1.0†††***	124.0 ± 1.3†††
20	115 ± 1	131.0 ± 2.2†††***	128.0 ± 1.0†††

Diastolic Blood Pressure			
Days	Control	CsA	CsA+DOPET
0	91.0 ± 1.5	96.2 ± 1.9	94 ± 2.5
5	93.8 ± 0.5	99.2 ± 1.1	100.0 ± 2.2
10	96.2 ± 0.4	107.2 ± 0.9†††***	104.0 ± 2.0†
15	94.5 ± 3.0	109.4 ± 0.4†††***	104.5 ± 1.9†
20	96.8 ± 2.3	114.0 ± 1.6†††***	107.7 ± 2.0††

Each group Vs Day 0: † *p* < 0.05 †† *p* < 0.01 ††† *p* < 0.001

CsA Vs Control: * *p* < 0.05 ** *p* < 0.01 *** *p* < 0.001

Fig. 6. Effect of CsA (15 mg/kg/day) and DOPET (20 mg/kg/twice daily) on systolic and diastolic pressure. The upper panel shows the systolic pressure trend during the treatment period. The lower panel shows the diastolic pressure results. †*P* < 0.05, ††*P* < 0.01, †††*P* < 0.001 versus day 0; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus control.

no detrimental effect, confirming the low toxicity of this antioxidant phenol. The lack of a protective effect of DOPET on blood pressure and kidney haemodynamics fits very nicely with the histological data indicating that the antioxidant was unable to act on the arteriopathy (Figure 5B and Table 2). Indeed, among the other lesions, arteriopathy is considered one of the major causes of reduced GFR and increased blood pressure following chronic CsA administration. It is highly unlikely that the lack of effect may be dependent on pitfalls in the experimental design, i.e. DOPET dosage. Our data in fact indicate that, at this dosage, DOPET completely reverses CsA-induced oxidative stress. Moreover, Visioli *et al.* reported that DOPET, at doses as low as 414 µg/kg, was able to protect against oxidative stress caused by passive cigarette smoke, as measured by the urinary excretion of 8-iso-PGF (2α) as an *in vivo* index of lipid peroxidation [37]. How can we explain these

in vivo results? The most reasonable interpretation of the present data is that the process leading to a blood pressure increase and GFR decrease is not necessarily related to the rise in free radicals. Thus, even when CsA-induced oxidative stress is completely reverted by DOPET, renal failure and hypertension cannot be prevented because of other underlying mechanisms, such as arteriopathy, an effect that appears independent of oxidative stress. Such a hypothesis seems to be in contrast with literature data showing that the administration of 'antioxidant drugs' like vitamin E [38] and lycopene [39] are able to reduce oxidative stress and ameliorate renal function after CsA treatment. It is highly likely that these two effects are mutually independent. Indeed, it should be underlined that both compounds, besides their antioxidant activity, exert key functions such as modulation of enzymatic activities and alteration of gene expression, which could account for their protective effect against

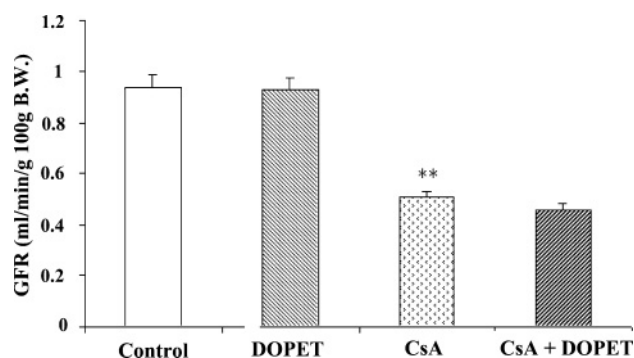


Fig. 7. Effect of DOPET on the glomerular filtration rate (GFR) measured by inulin clearance in CsA-treated rats. Inulin concentrations in plasma and urine were calculated by colorimetric method. *** $P < 0.0001$ versus control.

CsA treatment [22,40]. On the other hand, Zhong *et al.* have demonstrated that the overexpression of superoxide dismutase (SOD), by gene delivery, 3 days prior to the *in vivo* CsA administration, can partially reduce CsA-induced pathological alterations and inhibition of renal function [41]. However, also in this study, the modest protection of GFR (measured by a less accurate method like creatinine clearance) is not in agreement with the almost complete block of ROS formation, thus indicating that CsA-induced kidney injury is only partially due to oxidative stress. In this respect, the reported results are in agreement with our previously published data indicating that DOPET was able to completely prevent CsA-induced oxidative stress in rat tubular cells, but was ineffective in ameliorating the associated reduction of cell viability [21].

In conclusion, our data indicate that DOPET, a natural antioxidant of olive oil, is able to completely prevent artery and renal oxidative stress induced by CsA, and it has only a minor effect on the associated tubular injury and interstitial fibrosis without affecting the arteriolopathy. Finally, the same antioxidant is unable to ameliorate the increase in blood pressure and the reduction of GFR associated with CsA administration.

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Conflict of interest statement. None declared.

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