

Red Wine Polyphenols Prevent Cyclosporine-Induced Nephrotoxicity at the Level of the Intrinsic Apoptotic Pathway

R. REZZANI¹, S. TENGATTINI¹, F. BONOMINI¹, F. FILIPPINI¹, O. PECHÁŇOVÁ², R. BIANCHI¹, R. ANDRIANTSITOHAINA³

¹Division of Human Anatomy, Department of Biomedical Sciences and Biotechnology, University of Brescia, Brescia, Italy, ²Institute of Normal and Pathological Physiology, Centre of Excellence for Cardiovascular Research, Slovak Academy of Sciences, Bratislava, Slovak Republic, ³INSERM UMR 771, Angers; CNRS UMR 6214, Angers; Université d'Angers, UFR de Médecine, France

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Summary

Flavonoids, polyphenol derivatives of plant origin, possess a broad range of pharmacological properties. A number of studies have found both pro/anti-apoptotic effects for many of these compounds. For these reasons we investigated whether ProvinolsTM, flavonoids obtained from red wine, have anti-apoptotic properties. The investigations have been carried out in rats treated with Cyclosporine A (CsA). In particular, four groups of rats have been treated for 21 days with either olive oil (control group), with CsA, with ProvinolsTM, or with CsA and ProvinolsTM simultaneously. Oxidative stress, systolic blood pressure, body weight, biochemical parameters and different markers of pro/anti-apoptotic pathway were measured. CsA produced an increase of systolic blood pressure, a decrease in body weight, serum creatinine levels, urinary total protein concentration and creatinine clearance. Moreover, CsA induced renal alterations and the translocation of Bax and cytochrome c from cytoplasm to mitochondria and *vice versa*. These changes activated the caspase cascade pathway, that leads to morphological and biochemical features of apoptosis. ProvinolsTM restored morphological and biochemical alterations and prevented nephrotoxicity. In conclusion, this study may augment our current understanding of the controversial pro-/anti-apoptotic properties of flavonoids and their molecular mechanisms.

Key words

Apoptosis • Cyclosporine nephrotoxicity • Immunohistochemistry • Kidney disease • ProvinolsTM

Corresponding author

R. Rezzani, Department of Biomedical Sciences and Biotechnology, Division of Human Anatomy, University of Brescia,

Viale Europa 11, 25123, Brescia, Italy. Fax: +39 0303717486. E-mail: rezzani@med.unibs.it

Introduction

Plants have been used for the prevention and treatment of various human diseases throughout history. Some natural foods (vegetables, red wine, some other beverages and fruits) are considered to possess beneficial physiological effects such as antioxidant, anti-cancer, anti-aging, and anti-inflammatory effects. The polyphenols of plants in particular are major compounds with antioxidant effects (Yokozawa and Nakagawa 2004, Durak *et al.* 2007). In general, more than two thirds of the polyphenols consumed in the diet are flavonoids. Red wine flavonoids have generated considerable interest especially due to their *in vivo* and *in vitro* antioxidant capabilities. Their beneficial effects have been described mainly in relation to the French paradox phenomenon as well to the Mediterranean diet. In fact, the Mediterranean diet, rich in fruits and red wine, was shown to protect against the development of cardiovascular diseases and atherosclerosis (Carollo *et al.* 2007, Pecháňová *et al.* 2006). Another therapeutically relevant effect of flavonoids may be their ability to induce nitric oxide production and vasodilatation as well as the expression of genes that protect cardiovascular system (Curin and Andriantsitohaina 2005). Recently, the possible advantage of moderate wine consumption in patients with

chronic renal failure was hypothesized (Caimi *et al.* 2004) and the protective role of flavonoids on nephrotoxicity, induced by an immunosuppressive drug, have been shown. In particular, it has been demonstrated that ProvinolsTM, a flavonoid mixture isolated from red wine containing (in mg/g of dry powder) 480 proanthocyanins, 61 total anthocyanins, 19 free anthocyanins, 38 catechin, 18 hydroxycinnamic, 14 flavonols and 370 polymeric tannins (Pecháňová *et al.* 2006), was able to minimize renal side effects of cyclosporine A (CsA) treatment.

However, despite many studies that have been conducted on flavonoid functions, their antioxidant properties remain somewhat debatable, and the detailed molecular mechanisms of their effects remain largely unknown as well as their pro-/anti-apoptotic effects.

The objective of the present study was to assess the apoptosis-modulating effects of ProvinolsTM and to elucidate the molecular mechanisms underlying its activity on the apoptosis of renal parenchyma. The study has been carried out using an experimental model including the treatment with cyclosporine A, the most used immunosuppressive drug. This drug is normally used after solid-organ transplantation and in the treatment of several autoimmune diseases (Kahan 1989, de Mattos *et al.* 1996), but several renal and vascular toxic effects have been found to be associated with life-long treatment of transplanted patients with CsA and in experimental conditions (Burdmann *et al.* 2003, Rezzani 2004). The damage is related to tubulointerstitial fibrosis, mainly in proximal tubules and glomerular vasoconstriction (Justo *et al.* 2003, Rezzani *et al.* 2005). Moreover, several groups have identified an increased rate of tubular cell apoptosis both *in vitro* and *in vivo* indicating apoptosis as a mechanism of CsA nephrotoxicity (Hortelano *et al.* 2000, Ortiz *et al.* 1998, Yang *et al.* 2002). It has been shown that apoptosis induced by CsA is associated with the translocation of Bax to the mitochondria and that Bax antisense oligodeoxynucleotides is protected from CsA-induced apoptosis. CsA promoted a caspase-independent release of cytochrome c and Smac/Diablo from mitochondria and also led to a caspase-dependent loss of mitochondrial membrane potential. Caspase-2, caspase-3 and caspase-9 were activated and specific caspase inhibitor prevented apoptosis (Justo *et al.* 2003).

Here we studied renal cytoarchitecture and physiological functions as well as some markers of oxidative stress (Chi *et al.* 2005) and apoptotic pathway, such as superoxide dismutase (SOD), Bax, caspase-3 and

cytochrome c in animals treated with cyclosporine A and flavonoids.

Our findings indicate that apoptosis is regulated by ProvinolsTM underlying its role in the correction of CsA-induced both structural and functional changes of the kidney. Moreover, our data also add new insights into the controversies regarding the anti-/pro-apoptotic properties of dietary flavonoids.

Materials and Methods

Drugs and chemicals

Cyclosporine A (Sandimmun infusion concentrate 100 mg/ml) was obtained from Novartis (East Hanover, NJ, USA) and dissolved in olive oil to a final concentration of 50 mg/ml. ProvinolsTM (dry powder) has been supplied from the Société Française de Distilleries (Vallont Pont d'Arc, France).

Animals

Male Wistar rats (220±6 g) were used (Harlan, Italy). All animals were fed with standard rat chow and water *ad libitum* and kept in a temperature-controlled environment (20-22 °C) with an alternating 12 h light-dark cycle. The experimental protocol was approved and conducted in accordance with the Italian Ministry of Health and complied with the Guiding Principles in the Use of Animals in Toxicology.

Experimental protocol

Experiments were performed on four groups consisting of twenty rats each. The control group was treated with the CsA vehicle, olive oil (1 ml/kg, s.c.) for 21 days. The second group received CsA at a dose of 15 mg/kg/day, s.c., for 21 days. In the third group, rats were supplemented with ProvinolsTM (40mg/kg/day in the drinking water). To ensure that each rat received the complete dose of ProvinolsTM, the calculated amount of ProvinolsTM was given to each animal in the appropriate volume of water (0.2 mg/ml). Daily water consumption was controlled, with graduated Richter tubes, and ProvinolsTM concentration in the drinking fluid was adjusted, if necessary. The fourth group received ProvinolsTM concurrently during CsA injections for 21 days.

Measurements of body weight, systolic blood pressure

Body weight and systolic blood pressure (SBP) were measured weekly. SBP (initial value 97±3 mm Hg)

was measured with a tail-cuff technique (Apollo-2AB Blood Pressure Analyzer, Woodland Hills, CA, USA). Values for SBP were obtained by averaging readings from three measurements.

Collection of samples

At the end of the study, rats were housed individually in metabolic cages for a 24 h urine collection. Urine volumes were measured and urine samples were stored at -20°C until the determination of biochemical parameters. After animal decapitation under anesthesia, blood samples were obtained. One part was used to determine the concentration of drug in whole blood using a TDx/TDxFLx cyclosporine assay kit from Abbott Laboratories; the other part was centrifuged at 1800 g for 15 min at 4°C and sera were separated and stored at -20°C until assayed for creatinine. Kidneys were excised quickly, washed immediately with ice-cold physiological saline, blotted dry and weighed. Portions were taken for histopathological study and the remaining parts of the kidneys were homogenized in ice-cold saline, and were centrifuged at 3000 g for 10 min at 4°C . The supernatants were divided into aliquots and kept at -20°C until assayed for glutathione (GSH).

Measurements of kidney function

Serum creatinine was analyzed using a commercial kit from Sigma-Aldrich Diagnostics (St. Louis, MO, USA) and the urinary protein concentration was determined using a kit from Biomerieux (Marcy-l'Etoile, France). Glomerular filtration rate was calculated as creatinine clearance using standard formulae according to Shi *et al.* (2004).

Serum creatinine results are expressed as mg/dl, those of urinary protein concentration as g/dl and those of creatinine clearance as ml/min/kg, each of them being based upon at least 6 independent determinations.

Determination of renal GSH content

Renal GSH was determined as described previously by Dudek *et al.* (2005), using a colorimetric assay Bioxytech GSH-400 (Oxis Research, Foster City, CA, USA) according to the manufacturer's instructions. The absorbance was measured at 400 nm and the GSH concentration was calculated using a standard solution of GSH. Results are expressed as $\mu\text{mol/l}$.

Histopathological studies

The kidney portions taken for histopathological

studies were divided in two parts. One part was fixed in 10 % formalin, embedded in paraffin according to standard procedures and sectioned at $5\ \mu\text{m}$ by a microtome. These sections were deparaffinized, rehydrated and finally treated with hematoxylin-eosin and DNA fragmentation (TUNEL staining) for morphological studies and with caspase-3, SOD and Bax for immunohistochemical studies. The second part of each sample was frozen and used for histochemical study (cytochrome c).

DNA Fragmentation (TUNEL staining)

Kidney sections were labeled using TdT-Fragel DNA Fragmentation Detection Kit (TUNEL staining) according to manufacturer's instructions (Calbiochem, San Diego, CA). The number of TUNEL-positive cells was quantified by taking the average of 20 fields at x200 magnification.

Immunohistochemical localization of SOD, caspase-3 and Bax proteins

Before the immunohistochemical analysis, sections were deparaffinized, rehydrated, and then immersed in 3 % hydrogen peroxide (H_2O_2) in methanol for 30 min to block endogenous peroxidase activity. Sections were incubated for 60 min at room temperature in normal goat serum for SOD, caspase-3 and Bax (1:5; Dakopatts, Milan, Italy) and serially treated with respective rabbit polyclonal antibodies: anti-SOD (diluted 1:500; QED Bioscience Inc, San Diego, CA, USA), anti-caspase-3 (1:50; Cell Signaling Technology, Danvers, MA) and anti-Bax (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Thereafter, sections were washed in TBS 0.1 M, pH 7.4, and then incubated with a biotinylated secondary antibody (goat anti-rabbit, 1:50; Dakopatts, Milan, Italy) for 60 min and then with the avidin-biotin horseradish peroxidase complex (ABC kit; Dakopatts, Milan, Italy) for additional 60 min. Sections were then immersed in a solution of 0.05 % 3,3-diamino-benzidine tetrahydrochloride (DAB, Sigma-Aldrich, Milan, Italy) and 0.03 % H_2O_2 . Finally, sections were counterstained with hematoxylin, dehydrated, and mounted.

Cytochrome c histochemistry

Briefly, frozen samples were cut in a cryostat to a thickness of $5\ \mu\text{m}$. The sections were incubated for 1 h at 37°C with DAB 20 mg, acetate buffer 0.1 mol pH 5.5, MnCl_2 0.1 % and 0.1 ml H_2O_2 and then dehydrated, counterstained with hematoxylin and mounted according

Table 1. Effects of Provinols™ on body weight, blood pressure, kidney function, CsA blood level and GSH activities in CsA-treated rats.

	CsA	Control	Provinols™	CsA+Provinols™
Body weight (g)	242 ± 15*	312 ± 12	315 ± 9	298 ± 12
Systolic BP (mm Hg)	145 ± 3*	98 ± 4	96 ± 5	97 ± 3
Serum creatinine (mg/dl)	1.38 ± 0.32*	0.53 ± 0.27	0.65 ± 0.37	0.63 ± 0.33
Urinary protein (g/dl)	750 ± 23*	357 ± 12	389 ± 15	372 ± 20
Ccr (ml/min/kg)	1.4 ± 0.07*	3.1 ± 0.91	2.9 ± 0.12	3.0 ± 0.19
CsA blood level (µmol/l)	6.57 ± 1.42	0	0	6.35 ± 0.96
GSH (mmol/l)	0.31 ± 0.06*	2.47 ± 0.09	2.34 ± 0.08	1.3 ± 0.05

* p < 0.05 vs control.

For quantitative analysis, the 5-µm-thick sections including cortical and medullary region were evaluated at x100 magnification using an optical microscope (BX50; Olympus, Hamburg, Germany) equipped with an image analyzer (Image pro Plus; Milan, Italy). The integrated optical density (IOD) was calculated for arbitrary areas, measuring 10 fields with the same area for each sample. Data were pooled to represent a mean value, and a statistical analysis was applied to compare the results obtained from the different experimental groups.

Statistical analysis

Samples were analyzed and scored blindly. Values are expressed as the mean ± S.D. Data were analyzed using the ANOVA and Bonferroni test. $P < 0.05$ was considered significant.

Results

Effect of Provinols™ on body weight and blood pressure in CsA-treated rats

CsA treatment significantly reduced body weight as compared to control rats. Provinols™ alone did not affect body weight but completely prevented its decrease upon CsA treatment (Table 1). SBP was significantly increased at the end of CsA treatment from 98±4 mm Hg in control rats to 145±3 mm Hg in CsA-treated rats. Provinols™ completely prevented the rise in SBP induced by CsA treatment (Table 1).

Effect of Provinols™ on renal functions

CsA-induced nephrotoxicity was reflected by the significant increase in serum creatinine level and urinary total protein concentration, and by a decrease in creatinine clearance (Table 1). Provinols™ alone did not

significantly affect these parameters but it restored these values toward those of controls when it was administered with CsA. Thus, Provinols™ restored CsA-induced renal dysfunction. CsA blood levels were not affected by Provinols™ (Table 1).

Effect of Provinols™ on CsA-induced oxidative stress

Glutathione (GSH), an antioxidant enzyme, was significantly reduced after CsA treatment when compared to that of control rats. Provinols™ inhibited the CsA-induced depletion of GSH. Thus GSH concentration was not significantly different between control and Provinols™ plus CsA group (Table 1).

Histopathological studies – hematoxylin-eosin

Renal CsA damage was mainly observed in the cortical region and consisted of tubular and glomerular injury with dilation of the lumen and Bowman's capsule, respectively (Fig. 1A). On the contrary, kidneys from control, Provinols™ and CsA plus Provinols™-treated rats showed normal cytoarchitecture (Figs 1B, 1C and 1D).

DNA fragmentation (TUNEL staining)

The number of TUNEL-positive cells was high in CsA-treated rats (Fig. 2A) compared to those from control and Provinols™-treated animals (Figs 2B and 2C). Following 21 days of CsA plus Provinols™ treatment, TUNEL-positive cells were significantly reduced (Fig. 2D) compared to CsA group and it was similar to the control group (quantitative data - Fig. 2E).

Immunohistochemical studies for SOD, caspase-3 and Bax

CsA treatment significantly decreased SOD expression (Fig. 3A) in cortical renal parenchyma

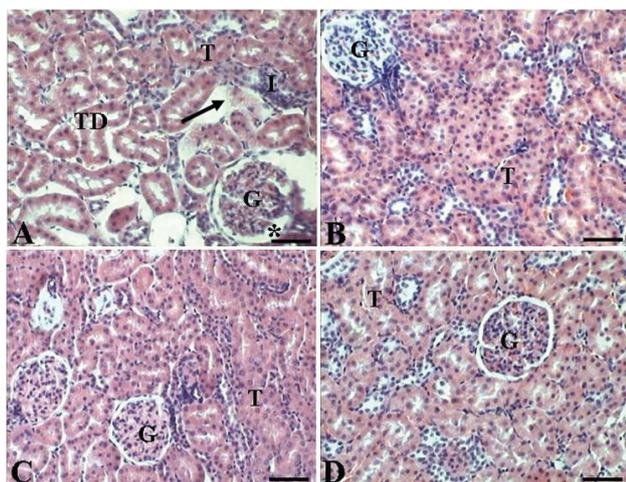


Fig. 1. Hematoxylin-eosin staining of renal cortex in rat: (A) CsA, (B) control, (C) ProvinolsTM, (D) CsA + ProvinolsTM (Bar: 50 μ m). T = tubules; G = glomeruli; TD = tubular dilation; I = infiltrates. The arrow shows renal fibrosis; the asterisk shows Bowman's capsule dilation.

compared to control and ProvinolsTM-treated rats (Figs 3B and 3C). CsA plus ProvinolsTM increased SOD expression toward that of control rats (Fig. 3D).

Figure 3G shows that CsA administration induced a huge caspase-3 expression when compared to the weak expression observed in control, ProvinolsTM and CsA plus ProvinolsTM-treated rats (Figs 3H, 3I and 3L). All data were confirmed also by quantitative analysis (Figs 3F and 3N).

Furthermore, Bax immunostaining, after CsA treatment, showed a granular positivity in tubular renal structures (Fig. 4A), whereas a high and diffuse positive staining was observed in renal tubules of control, ProvinolsTM and CsA plus ProvinolsTM-treated rats (Figs 4B, 4C and 4D).

Cytochrome c histochemistry

We observed a diffuse positivity after CsA treatment (Fig. 4F) in renal tubules and a "dot reaction product" in control, ProvinolsTM and CsA plus ProvinolsTM-treated animals (Figs 4G, 4H and 4I). This cytoplasmic positivity was evident in the epithelial cells of tubular structures.

Discussion

The flavonoids are a group of natural products currently receiving a great deal of attention. Several studies have reported that some flavonoids, including for instance kaempferol and quercetin, exert anti-oxidant effects and can also inhibit carcinogenesis (Cody 1988,

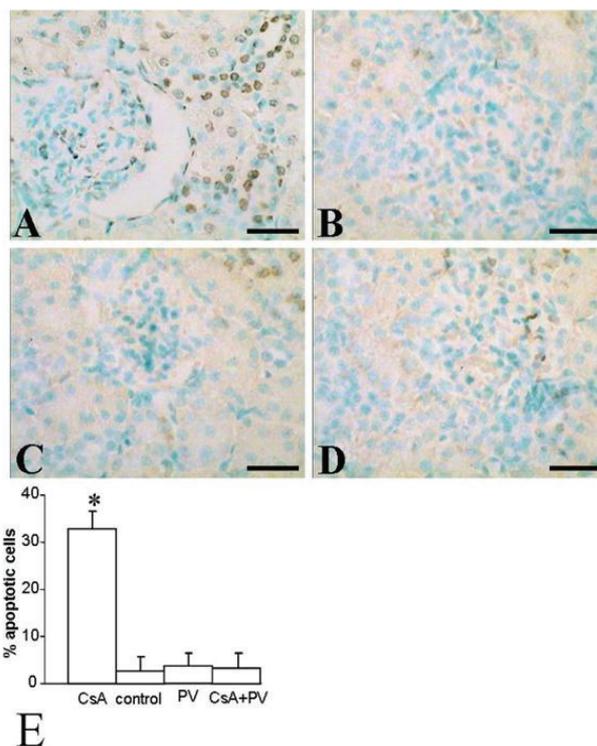


Fig. 2. TUNEL staining in renal cortex of rat: (A) CsA, (B) control, (C) ProvinolsTM, (D) CsA + ProvinolsTM (Bar: 30 μ m). (E) shows quantitative analysis (IOD) of apoptotic cells. *Statistical significant ($P < 0.05$) when compared with control.

Lee *et al.* 2005). Besides these antitumor activities, some flavonoids have been observed to exhibit a variety of beneficial biological activities, including anti-hypertensive, antiviral, and anti-inflammatory properties. Some flavonoids have also been reported to harbor anti-apoptotic properties (Shi *et al.* 2003). Despite the many studies conducted to determine the variety of biological functions associated with the flavonoids, the precise molecular mechanisms underlying their cellular effects remain largely unknown.

To the best of our knowledge, no previous studies have focused on the anti-apoptotic effects of ProvinolsTM, flavonoids from red wine. Here we found that ProvinolsTM: i) prevented the increase of SBP induced by CsA-treatment, and ii) improved renal function and prevented CsA-induced nephrotoxicity through an interaction with the apoptotic pathway.

ProvinolsTM and blood pressure

The mechanisms by which ProvinolsTM prevented blood pressure increase have not been assessed here, but many authors reported the antihypertensive effect of ProvinolsTM in different experimental models of hypertension. Administration of ProvinolsTM reduced the

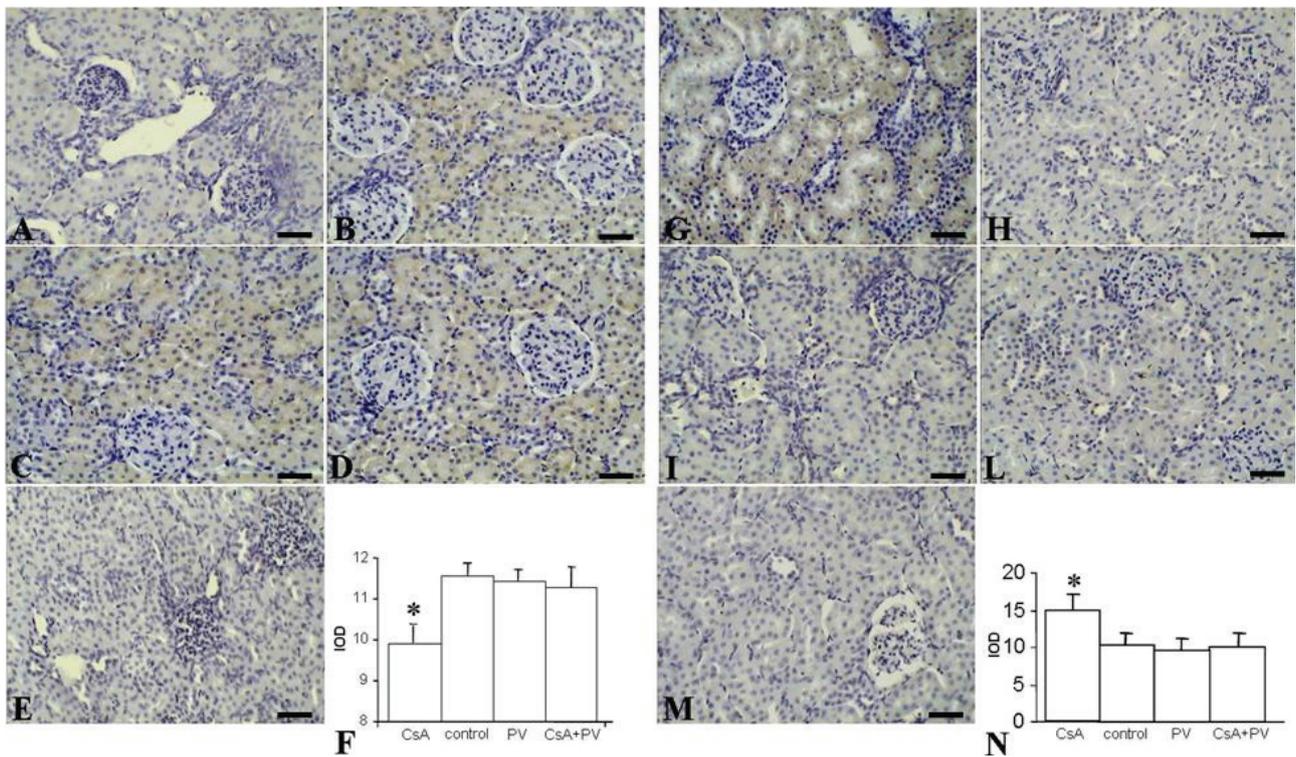


Fig. 3. Effect of Provinols™ on SOD (A-E) and caspase-3 (G-M) expression in CsA (A, G), control (B, H), Provinols™ (C, I), CsA + Provinols™ (D, L)-treated rats and in negative control (E, M) (Bar: 40 μm). (F, N) show quantitative analysis (IOD) of SOD and caspase-3 expression. * Statistically significant ($P < 0.05$) when compared with controls.

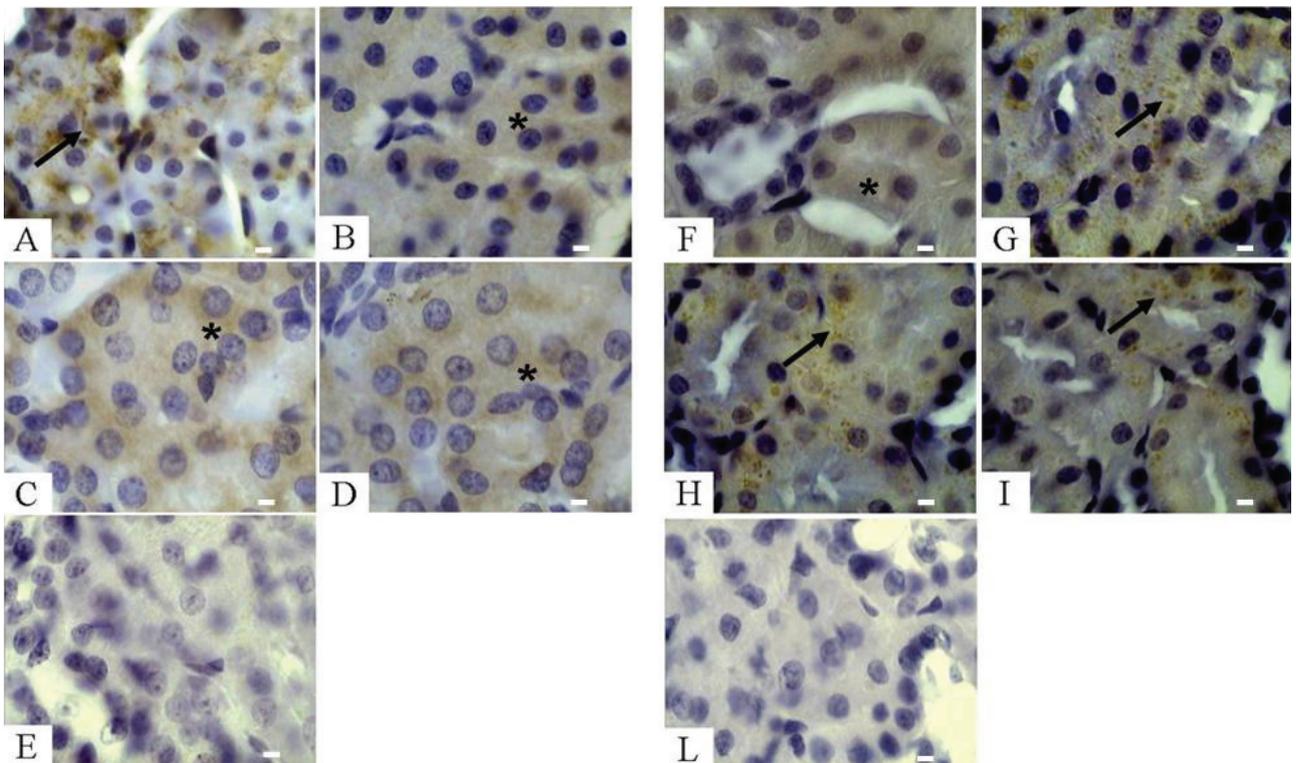


Fig. 4. Effect of Provinols™ on Bax (A-E) and cytochrome c (F-L) staining respectively in CsA (A, F), control (B, G), Provinols™ (C, H), CsA + Provinols™ (D,I)-treated rats and in negative control (E, L) (Bar: 4 μm). The arrows show the granular positivity; the asterisks show the diffuse positivity.

increase in both blood pressure and protein synthesis in the heart and aorta caused by chronic inhibition of NO synthesis (Bernátová *et al.* 2002, Pecháňová *et al.* 2004). This was accompanied by reduced end-organ damage such as myocardial fibrosis and aortic thickening. Provinols™ also prevented endothelial dysfunction that was associated with an increase of NO-synthase activity, a moderate enhancement of endothelial NO synthase expression and a reduction of oxidative stress in the left ventricle and aorta. Moreover, angiotensin II-induced hypertension, associated with endothelial dysfunction and an excessive NADPH oxidase-dependent vascular formation of reactive oxygen species (ROS), is prevented by administration of other red wine polyphenols (Sarr *et al.* 2006). Thus, the beneficial effects of Provinols™ in prevention of hypertension may result from their complex influence on the NO and ROS balance in the cardiovascular system. Therefore, the antihypertensive Provinols™ effect probably accounts at least partially for the reduced end-organ damage induced by CsA treatment in terms of nephrotoxicity. The latter properties of Provinols™ may protect the kidney against CsA-induced alterations in renal blood flow and kidney architecture, including tubular injury and interstitial fibrosis, especially at the level of the cortex, and therefore prevent the decrease of creatinine clearance.

Provinols™ and renal function

In this study we demonstrated that Provinols™ exerts its protective effects also through an interaction with the apoptosis pathway. In fact, evidence was provided that Provinols™ prevented Bax and cytochrome c translocation from cytoplasm to mitochondria and *vice versa* respectively. Once released, cytochrome c triggers the activation of caspase cascade activating the initiator caspases, such as caspase-3 induction. Finally, caspase-3, in turn cleaves various proteins leading to morphological and biochemical features characteristic for apoptosis (Gupta and Knowlton 2005, Orrenius 2004). Interestingly, Provinols™ treatment decreased caspase 3 activation and apoptosis. Our data showing that CsA-induced nephrotoxicity was linked to apoptosis, are in agreement with those reported in cultured renal tubular cells subjected to 3 h of ATP depletion with azide. Brooks *et al.* (2005) found that ATP depletion was mediated by a pathway involving Bax translocation, cytochrome c release, and caspase cascade activation. Moreover, they demonstrated that physiological Bax

immunoreactivity appeared as fine diffuse labeling in the cytoplasm and that, during apoptosis, its expression was observed as a granular labeling linked to the mitochondria. Furthermore, they suggested that granular cytochrome c immunopositivity, consistent with its expected mitochondrial localization in normal conditions, was detected as diffuse cytoplasmic positivity during apoptosis. Once released, cytochrome c activates the caspase cascade including caspase 3 and therefore apoptosis. In line with the present study we have shown that, delphinidin, an anthocyanin present in Provinols™ exerts an anti-apoptotic effect in endothelial cells through the NO guanylyl cyclase pathway, probably by upregulating endothelial NO synthase expression *via* the MEK1/2 pathway (Martin *et al.* 2003). Its preventive effect against cell death is associated with a strict control of calcium homeostasis and the inhibition of the release of cytochrome c from the mitochondria. Altogether, the anti-apoptotic effect of Provinols™, at least through endothelial protection, may contribute to the beneficial effects of natural dietary polyphenolic compounds, including those contained in red wine, against CsA-induced nephrotoxicity. Our data are in agreement with those reported by Orrenius *et al.* (2004) with regard to morphological alterations and an increase in apoptotic cell number. These effects are partially linked both to its antioxidant and anti-inflammatory properties. Indeed, Provinols™ enhanced the expression of the antioxidant enzymes (SOD and GSH) that, in turn, reduced ROS and oxidative stress. It is also possible that reduced ROS and oxidative stress reversed the intrinsic apoptotic pathway associated with mitochondria destabilization (Riles *et al.* 2006) following renal injury due to CsA-treatment.

In conclusion, this study may provide helpful information, allowing us to further address the controversial pro- /anti-apoptotic properties of various flavonoids. Our results also augment our current understanding of the molecular mechanisms underlying the differential cellular functions affected by flavonoid treatment.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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