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Quercetin reduces cisplatin nephrotoxicity in rats without compromising its anti-tumour activity

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Abstract

Background. Nephrotoxicity is the major limitation for the clinical use of cisplatin as an anti-tumoural drug. Our aim was to investigate the protective effect of quercetin on cisplatin nephrotoxicity in a rat tumour model *in vivo* and to examine the mechanisms of renal protection.

Methods. Breast adenocarcinoma (13762 Mat B-III) cells were inoculated subcutaneously in male Fischer rats and 7 days later, the rats were administered daily with quercetin [50 mg/kg/day, intraperitoneally (i.p.)] or vehicle. Four days after that, the rats were given a single dose of cisplatin (4 mg/kg, i.p.) or vehicle. Tumour growth and renal function were monitored throughout the experiment. Two or 6 days after cisplatin administration, the rats were killed and the kidneys and tumours were removed to examine renal function and toxicity markers in both tissues.

Results. In the kidney, cisplatin treatment induced: (i) a decrease in renal blood flow and glomerular filtration rate, (ii) tubular necrosis/apoptosis, (iii) increased lipid peroxidation and decreased endogenous antioxidant systems, (iv) increased expression of inflammation markers and (v) increased activity of the apoptosis executioner caspase-3. Cisplatin effectively reduced tumour size and weight.

Conclusions. Co-treatment with quercetin partially prevented all the renal effects of cisplatin, whereas it did not impair its anti-tumour activity. In conclusion, in a model of tumour-bearing rats, quercetin prevents the nephrotoxic effect of cisplatin without affecting its anti-tumour activity.

Keywords: acute kidney injury; cisplatin; nephrotoxicity; quercetin; renoprotection

Introduction

Cisplatin (cDDP; *cis*-diamminedichloroplatinum II) is a drug widely used against different types of solid tumours. However, its therapeutic utility is limited by acute and chronic nephrotoxicity. About 25% of patients receiving high-dose cisplatin undergo severe renal dysfunction. Cisplatin nephrotoxicity is chiefly characterized by tubular damage, mainly affecting the renal proximal and distal tubuli. Tubular damage may range from a mere loss of the brush border of epithelial cells to an overt tubular necrosis in severe cases [1]. Tubular damage causes impaired reabsorption, which underlies the observed proteinuria, hypomagnesemia and hypokalemia. In addition, cisplatin nephrotoxicity often progresses with reduced glomerular filtration rate (GFR) and increased serum creatinine [2, 3], which may result from the onset of the tubuloglomerular feedback mechanism and to reduced renal blood flow (RBF) resulting from renal vasoconstriction.

Although the mechanisms involved in cisplatin nephrotoxicity have been extensively studied, they are not yet fully elucidated. Recent studies suggest that cisplatin nephrotoxicity is a complex and multifaceted process in which cisplatin triggers cellular responses involving multiple pathways that culminate in renal damage and death [4, 5]. By inducing mitochondrial injury, cisplatin stimulates the production of reactive oxygen species (ROS) [6], which trigger an inflammatory response [7, 8] and several apoptotic signalling pathways mediated by caspases and mitogen-activated protein kinases [9–11].

Many different substances including antioxidants [4] have been investigated for their beneficial effects

on cisplatin-induced renal injury [12, 13]. However, most of these studies have been performed *in vitro* with cultured cells and in *in vivo* models using tumour-free animals. Because prospective renoprotective molecules may also affect the anti-tumour activity of cisplatin and also because tumours alter the metabolic and endocrine equilibrium [14, 15], protective strategies should be tested in tumour-bearing animals.

Quercetin is one of the most abundant flavonoids in the human diet and it is reported to exert many beneficial effects on human health including cardioprotection, anti-inflammatory, antiproliferative and anticancer activities derived from its antioxidant properties and other effects [16]. We previously reported that quercetin markedly prevented cadmium nephrotoxicity, through its strong capacity to inhibit oxidative stress and inflammation [17, 18]. Thus, the aim of the present study was to investigate the potential effect of quercetin on cisplatin nephrotoxicity in tumour-bearing rats, in which the influence of quercetin on anti-tumoural properties of cisplatin was also assessed.

Materials and methods

Chemicals and animals

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). Cisplatin was dissolved in 0.9% NaCl to a concentration of 1 mg/mL. Quercetin was dissolved in 0.9% NaCl containing 0.16% Tween-20 to a concentration of 50 mg/mL. All experiments were performed with male Fischer F344 rats weighing ~200 g (Charles River Laboratories). For the experiments, animals were housed in individual metabolic cages under controlled environmental conditions, fed with standard chow (PanLab, Barcelona, Spain) and allowed to drink water *ad libitum*. All procedures were approved by the Committee for Animal Care and Use of the University of Salamanca (Law 32/2007/Spain and RD 266/1998/CyL) and complied with the Guide for Care and Use of Laboratory Animals (Directive 2003/65/CE).

Preliminary experiments

On the basis of our preliminary and published data [18], we have performed a series of experiments to determine the optimum doses for the two drugs, cisplatin and quercetin. Cisplatin was injected at several doses (3.5, 4, 5 or 7.5 mg kg^{-1}). The regimen of 4 mg kg^{-1} significantly inhibited tumour growth and caused a marked nephrotoxicity, as assessed by functional and histologic measurements, without compromising the survival of the animals at end point. This regimen was used in our study. To obtain optimal concentrations of quercetin, we gave two doses of the drug (50 and 100 mg kg^{-1}). The 50 mg kg^{-1} dose was chosen because at a dose of 100 mg kg^{-1}, we did not observe further enhancement of the renoprotective effect.

Experimental model

All rats were injected in the dorsal area with 2×10^6 tumour cells (Rat 13762 Mat B-III breast adenocarcinoma; LGC Promochem, Barcelona, Spain). Seven days later, once tumours were visible and had grown to 100–200 mm^3, animals were randomly divided into four groups: 'control group (C)', animals that received physiological saline containing 0.16% Tween-20 (the vehicle for quercetin) daily for 9 days [100 μL/100 g, intraperitoneally (i.p.)], starting from Day 7 after tumour implant. 'Quercetin group (Q)' animals received quercetin (50 mg kg^{-1} once a day for 9 days i.p.), beginning from Day 7 after inoculation of tumour cells. 'Cisplatin group (P)' animals received a single dose of cisplatin (4 mg kg^{-1} i.p.), starting from Day 10 after tumour implant. 'Cisplatin–quercetin group (PQ)' animals received cisplatin on Day 10 after tumour implant and quercetin 4 days before and 5 days after cisplatin administration. Body weight and the physical activity were monitored daily; and blood and urine samples were collected every 2 days, beginning after chemotherapy treatment. At the end of the experiment (6 days after cisplatin administration), animals were anesthetized and tumour and kidneys were dissected, weighed and cut into two halves. One-half was fixed in 3.7% paraformaldehyde for histological examination. The other half was frozen in liquid nitrogen and stored at −80°C. Eventually, total tissue, nuclear and post-mitochondrial extracts were obtained from frozen tissue as described elsewhere [17, 19].

Renal function and tubular necrosis assessment

Creatinine, urea, sodium and potassium concentrations were measured in plasma and urine samples with an automatic analyser (Reflotron Plus; Roche Diagnostics, Barcelona, Spain). Creatinine clearance and fractional excretion of sodium were calculated using standard formulae [20]. Alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH) and *N*-acetyl glucosaminidase (NAG) enzymatic activities and protein concentration were measured in urine samples using automatic analysers (Reflotron® Plus; A. Menarini F360 model; A. Menarini Diagnostics, Firenze, Italy) with commercially available kits (Roche Diagnostics; A. Menarini Diagnostics). The kidney injury molecule 1 (KIM-1) protein was detected in urine and kidney tissue by western blot analysis using primary antibody anti-KIM-1/TIM-1 (goat 1:250; R&D Systems, Minneapolis, MN).

On Day 6 after cisplatin, [^{3}H] inulin and [^{14}C] *p*-aminohippuric (PAH) clearance was performed and GRF and RBF calculated as previously described by Morales *et al.* [19].

Tumour growth assessment

Tumour growth was monitored by caliper measurement every other day. Tumour volume was calculated according to the formula: $V = a \times b^2 \times 0.52$, where a is the largest superficial diameter and b is the smallest superficial diameter. Six days after cisplatin, tumours were removed, weighed and fixed in paraformaldehyde. For ethical reasons, animals were sacrificed before if tumours reached 3 cm.

Western blot

Western blot was performed with tissue extracts and urine samples as previously described [17, 21] using the antibodies described in each section. As appropriate in each case, after incubation with primary antibodies, the membranes were incubated with HRP-conjugated anti-goat (dilution 1:10000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit or anti-mouse IgG (1:10000; Bio-Rad Laboratories, Hercules, CA). The membranes were also reprobed with mouse monoclonal anti-β-actin (1:2000; Sigma-Aldrich) antibody to verify equal loading of protein in each lane.

Histopathological evaluation

Paraformaldehyde-fixed tissues were embedded in paraffin, and 3 μm tissue sections were stained with haematoxylin and eosin, examined and photographed under light microscopy. Renal damage was quantified by 'tubular atrophy', also called 'desepithelization', and hyaline casts scores'. Ten different fields of the renal tissue were examined in every slice, and the number of damaged tubuli and hyaline casts was determined in a blind fashion.

Assessment of oxidative status

Lipid peroxidation was measured as thiobarbituric acid-reactive substances by the colorimetric method of Recknagel [22]. Reduced and oxidized glutathione (GSH and GSSG) in post-mitochondrial supernatants were measured by the fluorometric method described by Hissin and Hilf [23]. Hydrogen peroxide (H_2O_2) and peroxidase activity in post-mitochondrial supernatants were measured using a commercial kit (Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit; Molecular Probes), according to the manufacturer's instructions.

Inflammatory mediators

Nuclear factor kappaB (NF-κB) activation was assessed by western blot analysis of the NF-κB p65 subunit in renal nuclear extracts (anti-NF-κB p65 1:1000 dilution; Santa Cruz Biotechnology) as previously described [17]. Renal intracellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1) and inducible nitric oxide synthase (iNOS) abundance in renal tissue was assessed by western blot [anti-ICAM-1 1:1000 dilution (Labgen, Frankfurt, Germany); anti-VCAM-1 1:1000 dilution (Santa Cruz Biotechnology) and anti-iNOS/NOS-2 1:1000 dilution (Santa Cruz Biotechnology)]. Tumour necrosis factor

alpha (TNF-α) concentration in kidney tissues was measured in triplicate by using the Rat TNF-α ELISA kit (R&D Systems), following the instructions provided by the manufacturer. Renal myeloperoxidase (MPO) activity was determined as previously described [24].

Detection of apoptosis

Nuclear DNA fragmentation in kidney and tumour tissue homogenates was quantitatively estimated by the Cell Death Detection ELISA kit (Roche Applied Science). Caspase activation was assessed by caspase-3/7 activity assay and western blot analysis of cleaved caspase-3 expression (anti-cleaved caspase-3, 1:500 dilution; Cell Signalling Technology, Danvers, MA) as detailed elsewhere [25]. Immunohistochemical analysis of cleaved caspase-3 was performed as previously described by Grande *et al.* [26].

Statistical analysis

All data were expressed as mean ± SEM. Multiple comparison among groups were analysed by one-way analysis of variance using the NCSS 2000 software, followed by individual comparisons with the Scheffe's Test. $P \leq 0.05$ was considered statistically significant.

Results

Quercetin reduces cisplatin nephrotoxicity in tumour-bearing rats

We first set up a model of cisplatin nephrotoxicity in Fischer F344 rats bearing a syngeneic tumour transplanted by injection of 13762 Mat B-III tumour cells. No mortality was observed in cisplatin-treated rats (or the other groups) at the end of the experiment. However, body weight was significantly lower in cisplatin-treated rats than in saline-treated rats (Table 1) indicating health deterioration. Quercetin was not able to reverse cisplatin-induced body weight loss. As shown in Figure 1A and B, on Day 2 after a single cisplatin injection, there was no significant difference in plasma creatinine and urea concentration among the groups, but this increase was already observed during Days 4, 6 and 8 after cisplatin injection. Accordingly, creatinine clearance was lower in cisplatin-treated than in saline-treated rats, indicating a reduction in GFR(Figure 1C). Six days after cisplatin injection, a significant reduction in inulin clearance further confirmed the reduced GFR. Furthermore, PAH clearance experiments revealed that cisplatin reduced renal plasma flow and filtration fraction and increased renal vascular resistance (RVR; Figure 1D-G**)**. Additionally in the cisplatin group, urinary volume, proteinuria, fractional excretion of sodium and potassium, urinary activities of NAG, LDH, ALP and GGT and urine and kidney content of KIM-1, a biomarker of tubular injury, were markedly increased with respect to the control group (Table 1). All these are later suggestive of tubular damage and tubular dysfunction. The alteration caused by cisplatin in all these parameters was significantly attenuated by cotreatment with quercetin, although treatment with quercetin alone had no effect (Table 1 and Figure 1). Histological studies revealed that kidneys obtained from animals treated with cisplatin had serious tubular damage, mainly in the corticomedullary junction, characterized by tubular dilation, necrosis, vacuolization and formation of hyaline casts (Figure 2), while glomeruli were not apparently affected. Co-treatment with quercetin reduced the number of hyaline casts and the extent of tubular necrosis induced by cisplatin in the corticomedullary area (Figure 2D and E).

Quercetin does not interfere with the anti-tumour activity of cisplatin

After characterizing the nephroprotective effect, we then ascertained whether or not quercetin modified the anti-tumour activity of cisplatin. In control and quercetin-treated animals, there was a considerable increase in tumour volume during the experiment. Administration of a single dose of cisplatin resulted in a significant reduction in tumour volume (Figure 3A). The decrease in tumour volume was similar in cisplatin + quercetin treated animals than in animals treated with cisplatin alone (Figure 3A). Tumour weight in animals treated with cisplatin and cisplatin + quercetin was markedly lower than that of control (untreated) rats, and no differences between animals treated with cisplatin alone and animals treated with cisplatin + quercetin were found (Figure 3B).

Examination of tumour tissue sections stained with haematoxylin and eosin revealed normal neoplasm

Table 1. Effect of quercetin on renal injury markers in cisplatin-induced nephrotoxicity[a]

	Control	Quercetin, (50 mg kg^{-1})	Cisplatin, (4 mg kg^{-1})	Cispl + Q
Body weight (g)	238 ± 5	227 ± 4	175 ± 4*	170 ± 4*
Urinary volume (mL/min)	6.3 ± 2	4.5 ± 1	17.4 ± 2*	19.0 ± 2*
Kidney index (%)	0.18 ± 0.02	0.17 ± 0.02	0.47 ± 0.03*	0.25 ± 0.03$^{\&}$
Proteinuria (mg/24 h)	24.3 ± 3	24.4 ± 4	76.5 ± 14 *	76.5 ± 14*
FE_{Na+} (%)	1.25 ± 0.16	0.71 ± 0.21	15.47 ± 2.88*	7.34 ± 0.81$^{*\&}$
FE_{K+} (%)	155.41 ± 20.03	73.03 ± 18.98	658.04 ± 118.0*	380.9 ± 58.4$^{*\&}$
Urinary NAG (U/24 h)	0.54 ± 0.05	0.48 ± 0.06	3.15 ± 0.30*	0.78 ± 0.06$^{\&}$
Urinary LDH (U/24 h)	66.50 ± 8.46	76.50 ± 6.65	105.00 ± 17.91*	67.25 ± 6.44$^{\&}$
Urinary ALP (U/24 h)	7.43 ± 1.02	4.38 ± 1.52	144.18 ± 24.86*	24.61 ± 3.39$^{\&}$
Urinary GGT (U/24 h)	1.59 ± 0.11	1.43 ± 0.12	30.62 ± 5.76*	13.54 ± 2.72$^{*\&}$
Urinary Kim-1 (% of control)	100.00 ± 20.87	217.14 ± 47.69	645.26 ± 49.57*	330.08 ± 67.99$^{*\&}$
Renal Kim-1 (% of control)	100.00 ± 5.73	94.61 ± 23.40	877.46 ± 210.06*	371.48 ± 143.33$^{*\&}$

[a]Values are expressed as mean ± SEM ($n = 9$ rats per group). Data were analysed using one-way analysis of variance followed by Scheffe's Test. Statistical significance: *$P < 0.05$ versus control group, $^{\&}P < 0.05$ versus cisplatin group. Tumour-bearing rats were treated with vehicle (control, C); quercetin alone (quercetin, Q); cisplatin treatment alone (cisplatin, Q) or cisplatin with pre-treatment of quercetin (cisplatin-quercetin, cispl + Q). FE_{Na+}, sodium fractional excretion; FE_{K+}, potassium fractional excretion; NAG, enzyme *N*-acetyl-beta-D-glucosaminidase (E.C.3.2.1.30); LDH, lactate dehydrogenase (E.C. 1.1.1.27); ALP, alkaline phosphatase (E.C. 3.1.3.1); GGT, gamma-glutamyl transpeptidase (E.C.2.3.2.2).

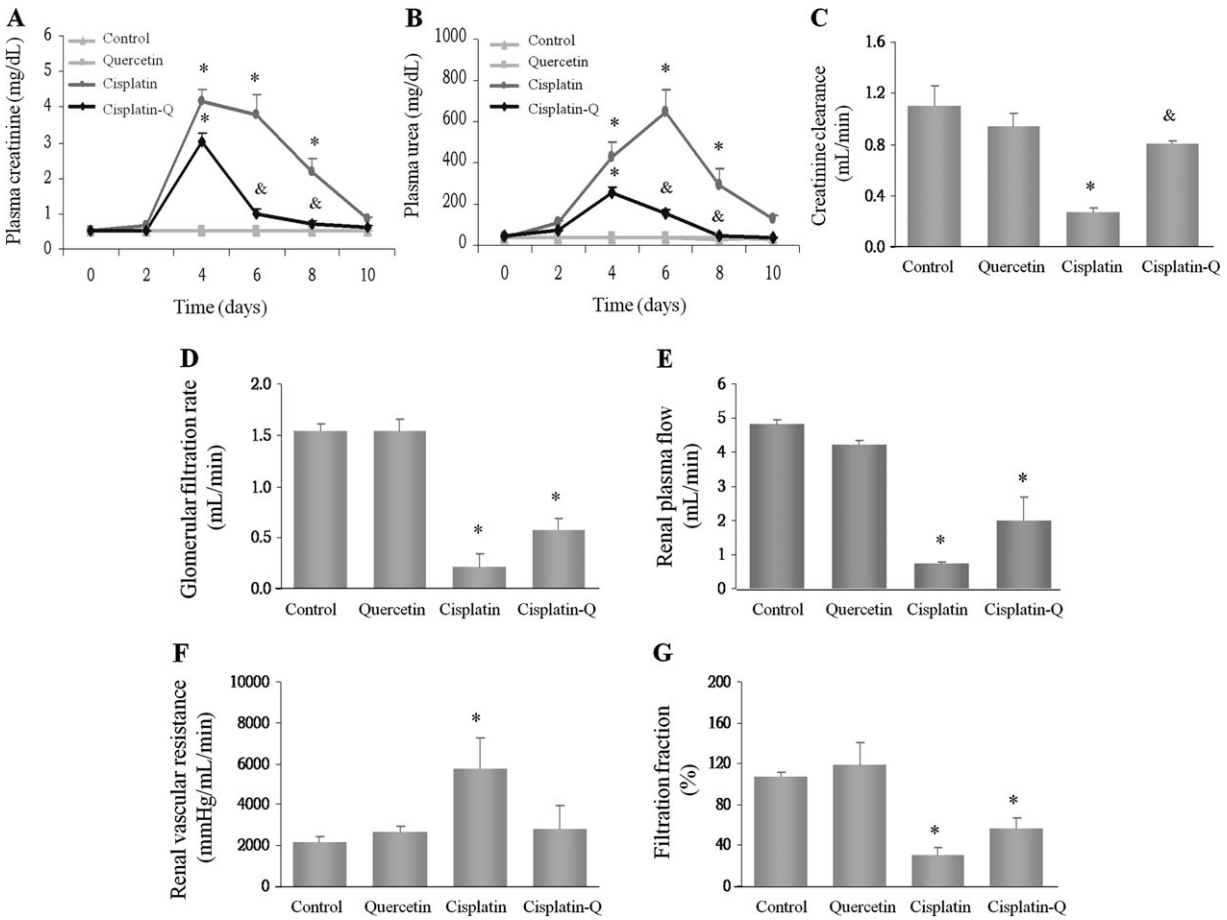

Fig. 1. Effect of quercetin on renal function in cisplatin nephrotoxicity. Nephrotoxicity was induced by intraperitoneal injection of cisplatin (4 mg kg^{-1}). Tumour-bearing rats were administrated quercetin (50 mg kg^{-1}) intraperitoneally once a day during 9 days, 4 days before and 5 days after cisplatin injection. Blood samples for plasma creatinine (**A**) and urea (**B**) determinations were collected the day of cisplatin administration and later every 2 days. At the end of the treatment period (Day 6 after cisplatin injection), creatinine clearance (**C**) and hemodynamic parameters (**D**–**G**) were evaluated. Control rats were injected with the vehicle of quercetin (0.9% NaCl + 0.16% Tween-20). Values are expressed as mean ± SEM ($n = 9$ rats per group, of which only 4 animals were used for acute clearance studies) from three independent sets of experiments. *$P < 0.05$ versus control group, &$P < 0.05$ versus cisplatin group. Animals were treated with vehicle, control; quercetin alone, quercetin; cisplatin treatment alone, cisplatin and cisplatin with pretreatment of plus quercetin, cisplatin-Q.

histology, with predominantly viable, highly proliferating cells and scarce necrotic areas (Figure 3C-a and Figure 3C-b). In contrast, sections from cisplatin and cisplatin + quercetin-treated tumours were characterized by large areas of necrosis, cell dilation, loss of cellular structures and rupture of tissue homogeneity. Tumour cells were swollen with enlarged and hyperchromatic nuclei. Perinuclear cytoplasm was condensed and eosinophilic. Moreover, cell density decreased and the number of giant, multinucleated cells dramatically increased (Figure 3C-c and Figure 3C-d). No histological differences were observed between tumours from cisplatin and cisplatin + quercetin-treated animals.

Quercetin reduces the renal oxidative stress caused by cisplatin

Once we learned that quercetin reduced the nephrotoxicity of cisplatin without affecting its anti-tumour properties, we went deeper into the underlying mechanism of protection. In this sense, because oxidative stress is a central pathological mediator of the renal damage caused by cisplatin [13, 27], we closely determined the antioxidant effect of quercetin. Six days after cisplatin administration, rats showed a significant increase in lipid peroxidation, as evidenced by a higher malondialdehyde content in kidney homogenates. This increase was markedly reduced by pre-treatment with quercetin (Figure 4A**)**. In comparison with the control group, kidneys from cisplatin-treated rats showed a significantly lower GSH/GSSG ratio (Figure 4B), derived from a lower GSH content and higher GSSG level (data not shown). Quercetin administration was able to restore the GSH/GSSG ratio to normal values (Figure 4B). Hydrogen peroxide (H_2O_2) levels were higher and peroxidase activity was lower in kidney tissue from cisplatin-treated rats compared with the control group (Figure 4C and D). Kidneys from quercetin + cisplatin-treated animals showed significantly

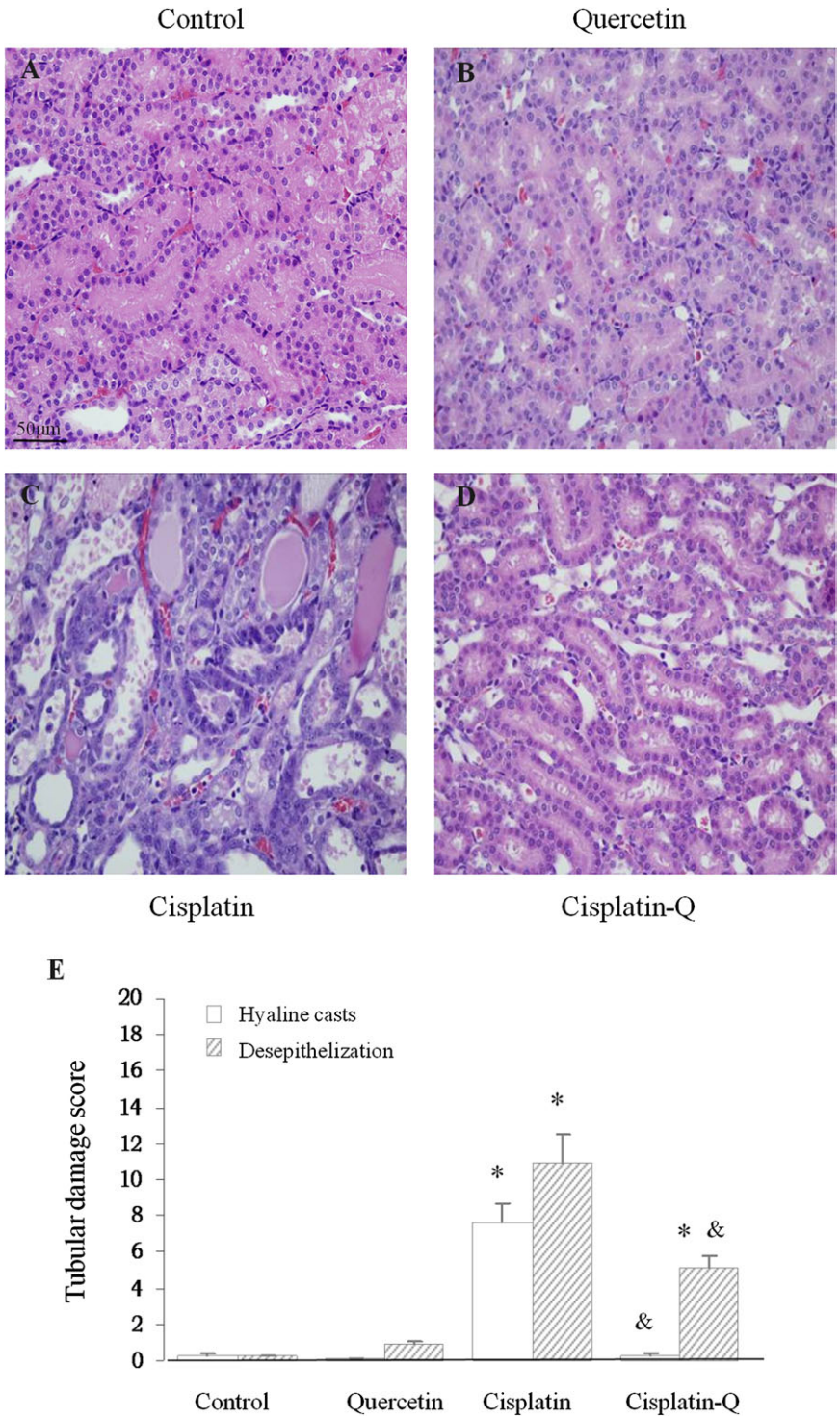

Fig. 2. Effect of quercetin on renal histology in cisplatin-induced nephrotoxicity. Histopathological analysis (**A**–**D**) and quantitative score of renal histological changes (**E**) at Day 6 after cisplatin administration in tumour-bearing rats. Animals were treated with vehicle (control); quercetin alone (quercetin); cisplatin alone (cisplatin) or cisplatin and quercetin (cisplatin-Q). Kidney sections were stained with haematoxylin and eosin and observed under ×40 magnification. The cisplatin-treated kidneys (C) showed marked injury with hyaline cast formation, sloughing of tubular epithelial cells, loss of brush border and dilation of tubules. These changes were less pronounced in quercetin pre-treated rats (D). Quercetin treatment alone (B) showed normal kidney architecture and histology. Data represent mean ± SEM ($n = 5$ rats per group) from three independent set of experiments. *$P < 0.05$ versus control group, &$P < 0.05$ versus cisplatin group.

lower hydrogen peroxide levels and higher peroxidase activity than animals treated with cisplatin alone (Figure 4C and D). Rats treated with quercetin alone did not show significant differences with respect to control rats in renal oxidative stress parameters (Figure 4C and D).

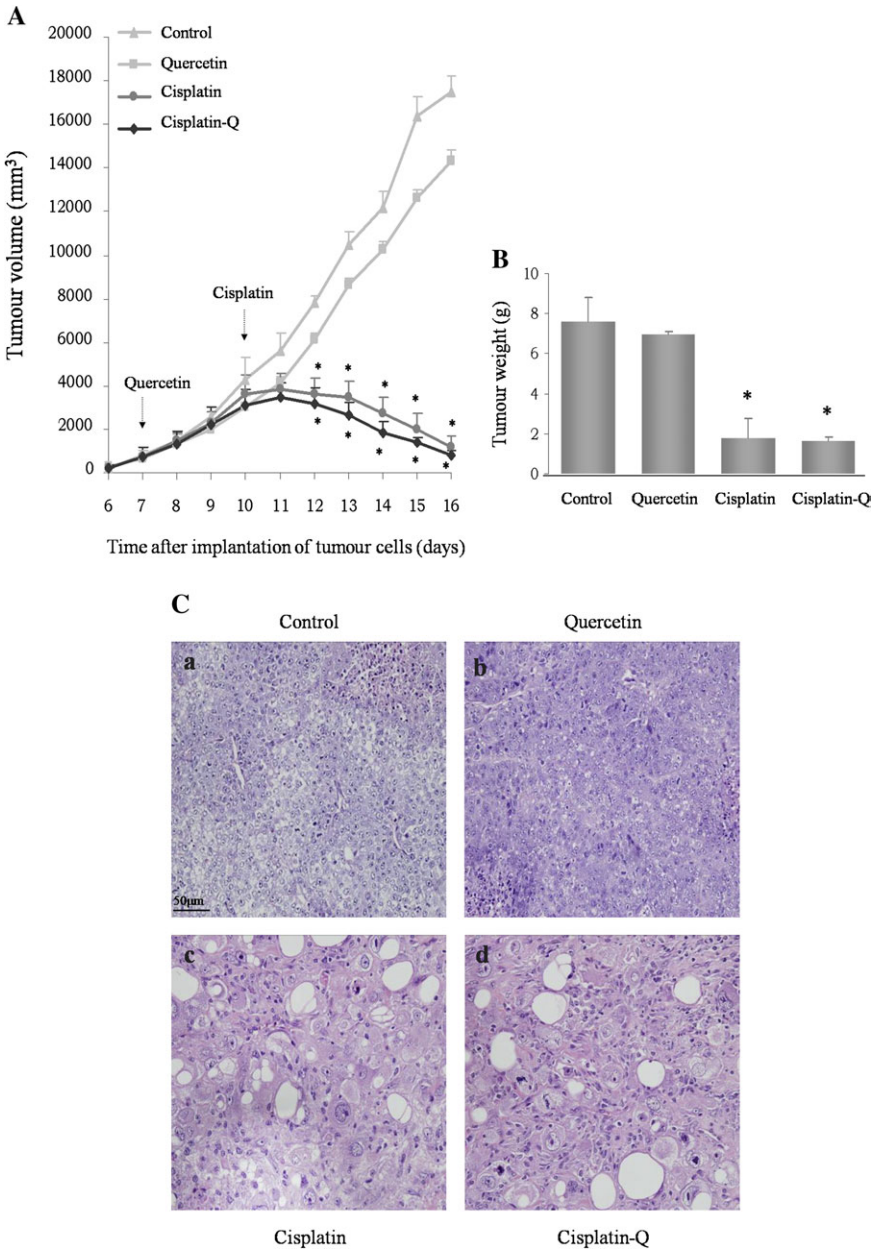

Fig. 3. Effect of quercetin on the anti-tumour activity of cisplatin. Tumour growth curves (**A**) and tumour weight (**B**) formed in rats 16 days after subcutaneous implantation of 13762 Mat B-III cells ($n = 9$ rats per group). Animals were treated with vehicle, control (-▲-); quercetin alone, quercetin (-■-); cisplatin treatment alone, cisplatin (-●-) and cisplatin plus quercetin, cisplatin-Q (-♦-). Histopathological analysis (**C**) of tumour tissues at Day 6 after cisplatin administration ($n = 5$ rats per group). Tumour sections were stained with haematoxylin and eosin and observed under ×40 magnification. Cisplatin-treated tumour (c) showed pronounced cell pleomorphism and functional differentiation as demonstrated by multinucleated giant cells, decreased cell density and higher level of necrosis–apoptosis as a sign of therapeutic efficacy. These changes were similar in cisplatin + quercetin rats (d). Quercetin treatment alone (b) showed normal neoplasm architecture and histology. Results are expressed as mean ± SEM from three independent sets of experiments. *$P < 0.05$ versus control group.

Quercetin reduces cisplatin-induced renal inflammation

Inflammation [28, 29] and the innate immune response [8] have been shown to play a significant role in cisplatin nephrotoxicity. As cisplatin-induced inflammatory damage is a very early event [9, 30], we evaluated the inflammatory process 2 days after cisplatin administration. We studied the effect of quercetin on several mediators and players of the renal inflammation and leucocyte infiltration observed after treatment with cisplatin. Particularly, we measured the renal levels of the proinflammatory cytokine TNF-α (Figure 5B) as well as the participation of intracellular mediators such as the activation of NF-κB (amount of p65 subunit in nuclear extracts from renal tissue; Figure 5A) and iNOS expression (Figure 6D). As an indication of cell infiltration, we determined the renal level of ICAM-1 and VCAM-1 (Figure 6A and B) and renal MPO, as a marker of leucocyte/

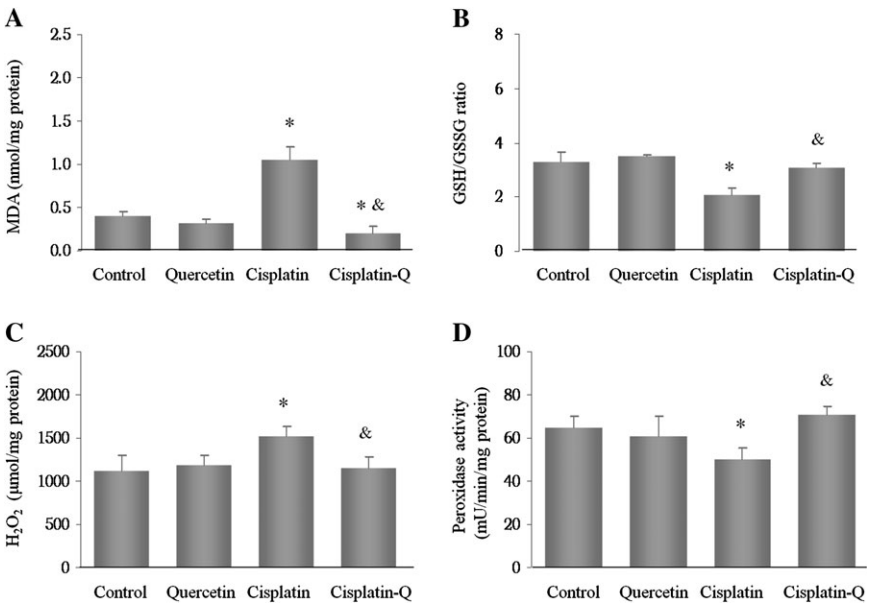

Fig. 4. Effect of quercetin on cisplatin-induced renal oxidative stress. Concentration of MDA (**A**), GSH/GSSG ratio (**B**), content of H_2O_2 (**C**) and peroxidase activity (**D**) were measured in kidney tissue at Day 6 after cisplatin administration in tumour-bearing rats. Animals were treated with vehicle (control); quercetin alone (quercetin); cisplatin alone (cisplatin) or cisplatin and quercetin (cisplatin-Q). Results are expressed as mean ± SEM ($n = 9$ rats per group) from three independent sets of experiments. *$P < 0.05$ versus control group, &$P < 0.05$ versus cisplatin group. MDA, malondialdehyde; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide.

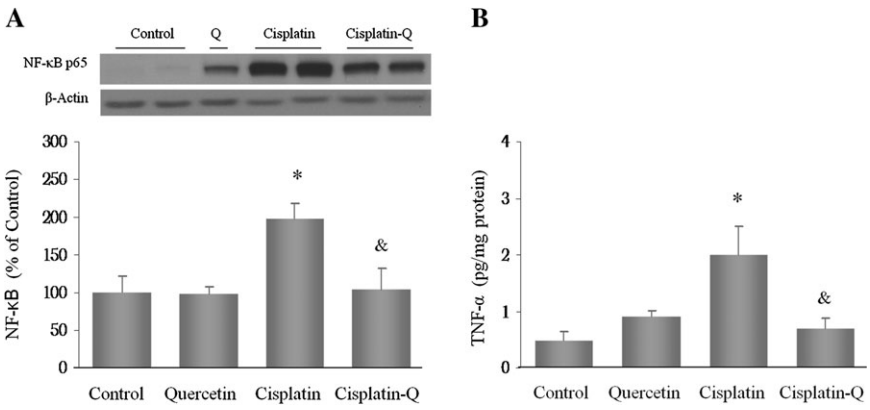

Fig. 5. Effect of quercetin on NF-κB p65 activation and TNF-α levels in cisplatin nephrotoxicity. Kidney tissue nuclear translocation of NF-κB p65 by western blot analysis and corresponding densitometric analysis at Day 2 after cisplatin administration in tumour-bearing rats. Results were normalized with respect to β-actin, used as load control. Data from NF-κB p65 expression were expressed as per cent increase compared with the control group (**A**). Concentration of TNF-α in kidney tissue (**B**). Animals were treated with vehicle (control); quercetin alone (quercetin); cisplatin alone (cisplatin) or cisplatin plus quercetin (cisplatin-Q). All values are expressed as mean ± SEM ($n = 5$ rats per group) from three independent sets of experiments. *$P < 0.05$ versus control group, &$P < 0.05$ versus cisplatin group. NS, non-specific; Comp, cold competition control; TNF-α, tumour necrosis factor alpha.

macrophage infiltration; Figure 6C. All these parameters were increased in animals treated with cisplatin with respect to untreated (control) animals. Quercetin did not exert any significant effect when administered alone but markedly reduced or normalized the alterations caused by cisplatin (Figures 5 and 6).

Quercetin inhibits cisplatin-induced renal apoptosis

Tubular necrosis resulting from epithelial tubular cell death is the central pathological event in cisplatin nephrotoxicity. As apoptosis has been repeatedly reported to occur in renal tubuli following cisplatin administration [31, 32], we evaluated the effect of quercetin on the extent of renal apoptosis induced by cisplatin. For this purpose, we determined the effect of cisplatin and quercetin on apoptotic hallmarks such as executor caspase activation and internucleosomal DNA fragmentation. As shown in Figure 7A, immunohistochemical analysis of renal tissue sections reveals that cisplatin induces a marked increase in the number of cells stained for cleaved caspase-3 within damaged tubular epithelium or shed into the tubular lumen. Co-treatment with quercetin together with cisplatin produced a dramatic

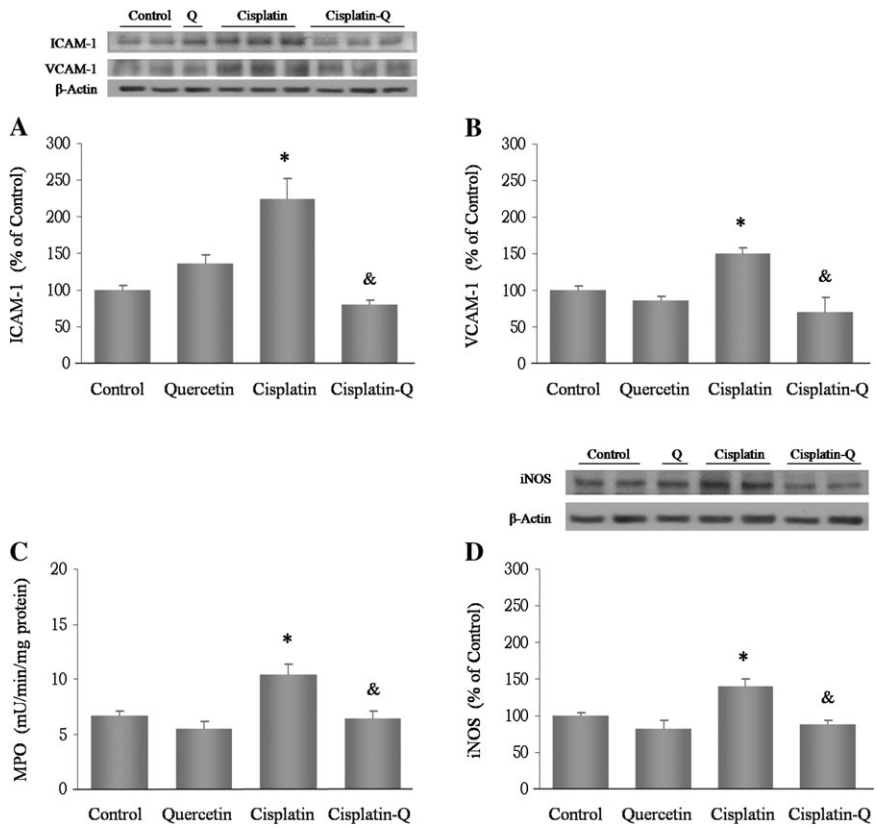

Fig. 6. Effect of quercetin on the expression of NF-κB-dependent proinflammatory molecules and MPO activity in cisplatin nephrotoxicity. Representative images of western blot and corresponding densitometric analysis of adhesion molecules ICAM-1 and VCAM-1 (**A** and **B**) and iNOS (**D**) proteins in kidney tissue 2 days after cisplatin administration in tumour-bearing rats. Results were normalized with respect to β-actin, used as load control. Neutrophil infiltration was assessed by measuring MPO activity in kidney tissue (**C**). Animals were treated with vehicle (control); quercetin alone (quercetin, Q); cisplatin alone (cisplatin) or cisplatin plus quercetin (cisplatin-Q). Data were expressed as percent increase compared with the control group and represent mean ± SEM ($n = 5$ rats per group) from three independent sets of experiments. *$P < 0.05$ versus control group, &$P < 0.05$ versus cisplatin group. ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cellular adhesion molecule-1; iNOS, inducible nitric oxide synthase.

decrease in the number of cells stained for cleaved caspase-3 (Figure 7A). Consistent with the immunohistochemistry results, western blot analysis of renal tissue extracts revealed that the level of cleaved caspase-3 was significantly higher in the kidneys of rats treated with cisplatin compared with the expression in the kidneys from control-untreated rats. Again, kidneys from cisplatin + quercetin-treated rats present lower levels of activated caspase-3 than those treated with cisplatin alone (Figure 7B). Furthermore, cisplatin increased DEVDase (caspase-3/7) activity in renal tissue extracts, which was prevented by co-administration of quercetin (Figure 7C). Finally, we observed that cisplatin significantly augmented the level of renal tissue DNA fragmentation, which was reduced by the co-administration of quercetin (Figure 7D). All these parameters were not altered by single treatment with quercetin, compared to controls (Figure 7).

Discussion

Worldwide, nephrotoxicity poses a considerable health and economic burden. Nearly 25% of the top 100, most used drugs in intensive care units are potentially nephrotoxic [33]. Moreover, nephrotoxicity causes 10–20% of the acute renal injury (AKI) cases [33]. AKI is a very serious condition with high incidence and mortality rate, which is estimated at ~50% of the cases despite dialysis treatment, especially within critically ill patients. Mortality increases to 80% when AKI progresses with multiorgan damage [35–37]. The clinical handling of renal injury and AKI is difficult and expensive because, other than dialysis, there are no available treatments. For this reason, the search for strategies to prevent nephrotoxicity constitute an active area of investigation. In addition to drug targeting and medical chemistry for new and safer molecules, a line of interest is the identification of renoprotective adjuvants for co-administration along with potentially nephrotoxic drugs, such as platinated antineoplastics.

At the preclinical level, many chemically unrelated antioxidants have been shown to protect the kidneys from cisplatin nephrotoxicity, especially in experimental animal models. They include curcumin, *N*-acetylcysteine, naringenin, selenium, vitamin C, vitamin E and other dietary components that scavenge free radicals formed by exposure to cisplatin [28, 38–40]. Although promising, antioxidants have not yet demonstrated a clear benefit in the clinical research conducted so far [41], which requires

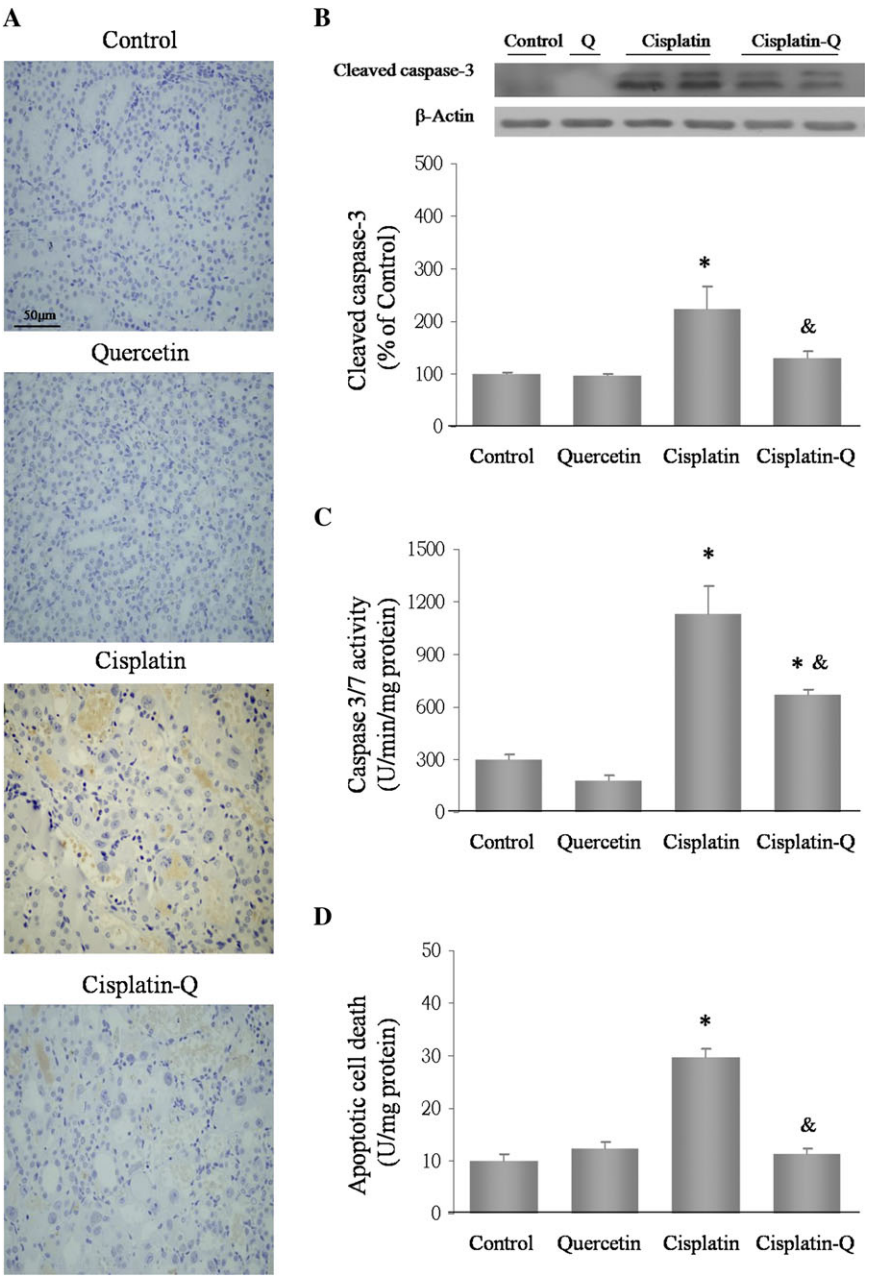

Fig. 7. Effect of quercetin on cisplatin-induced renal apoptosis. (**A**) Immunostaining for cleaved caspase-3 in kidney tissue of tumour-bearing rats 2 days after cisplatin administration. Kidney sections were observed under ×40 magnification. The expression of cleaved caspase-3 is highest in rats treated with cisplatin. The expression of cleaved caspase-3 was increased by cisplatin in the cytoplasm of tubular cells. Pre-treatment with quercetin decreased the renal expression of active caspase-3 after cisplatin injection. (**B**) Western blots and corresponding densitometric analysis of cleaved caspase-3 in kidney tissue. Results were normalized with respect to β-actin, used as load control, and data were expressed as per cent increase compared with the control group. (**C**) DEVDase (caspase 3/7) activity in kidney tissue 2 days after cisplatin administration in tumour-bearing rats. (**D**) Quantification by enzyme-linked immunosorbent assay of internucleosomal DNA fragmentation in kidney tissue. Animals were treated with vehicle (control); quercetin alone (quercetin, Q); cisplatin alone (cisplatin) or cisplatin plus quercetin (cisplatin-Q). Data were expressed as per cent increase compared with the control group and represent mean ± SEM ($n = 5$ rats per group) from three independent sets of experiments. *$P < 0.05$ versus control group, &$P < 0.05$ versus cisplatin group.

further investigation. In this line, a preclinical selection of candidates to be assayed at the clinical level must be pursued in order to (i) improve the efficacy of the preclinical to clinical transition and (ii) to reduce early failure rate in clinical assays through the drug discovery process. In this sense, it is necessary to test renoprotective candidates in preclinical studies also for their innocuousness on the anti-tumour effect of anti-neoplastics. Our results indicate that our experimental model of cisplatin administration in tumour-bearing rats is adequate to assess both nephrotoxicity and anti-tumour efficacy, as well as the underlying patho-physiological events, and also to test renoprotective strategies.

Quercetin has been reported as a renoprotective agent in cisplatin-induced nephrotoxicity *in vitro* [42] and *in vivo* models [30, 43]. However, these reports have only shown beneficial functional and histopathological effects in cultured tubular cells and tumour-free animals, and they have not assessed if quercetin interferes with the anti-tumour effect of cisplatin. Therefore, our study was designed to deeply examine the protective effect of quercetin on the underlying mechanisms of cisplatin-induced nephrotoxicity and, simultaneously, the effect of this bioflavonoid on the anti-tumour activity in tumour-bearing rats. Our results indicate that the natural flavonoid quercetin protects the kidneys from the toxic damage inflicted by the anti-cancer drug cisplatin, without altering its therapeutic anti-tumour activity. Specifically, quercetin showed a markedly decreased excretion of NAG, LDH, ALP, KIM-1 and GGT, thus suggesting a decreased tubular damage. These biomarkers are present in the tubular epithelial brush border, and the urinary excretion of them represents tubular necrosis or loss of the brush border thus have been widely used to assess tubular toxicity. NAG is a proximal epithelium intralysosomal membrane-bound enzyme, which is released into urine when disruption of lysosomal membranes occurs. KIM-1 is associated with early pathophysiological events underlying the incipient AKI [18, 20], thus suggesting that quercetin strikes at the very root of the mechanisms of damage, because it is capable of reversing most (if not all) alterations produced by cisplatin, rather than palliating a specific event or group of events by just targeting a specific pathological mechanism. The nephroprotective effect of quercetin seems to be related with its antioxidant activity and also with its capacity to inhibit renal inflammation and tubular cell apoptosis. Indeed, co-treatment with cisplatin plus quercetin partially but significantly preserves not only the integrity of the renal parenchyma but also GFR, RBF and renal excretory function. Very recently, it has been reported that cilastatin is able to protect primary cultures of proximal tubular cells from the cytotoxic effect of cisplatin, without modifying its cytotoxic effects on tumoural cells (HeLa) [44]. However, this study has been performed only '*in vitro*'.

Preservation of tubular integrity by quercetin through direct cytoprotective effects as well as indirectly, via attenuation of inflammation, may explain the partial tubular function maintenance observed upon co-treatment with cisplatin. In principle, preservation of tubular integrity would inhibit the reduction of GFR mediated by the tubuloglomerular feedback. Furthermore, because inflammation is known to induce renal vasoconstriction and to reduce RBF and GFR [45], prevention of inflammation should also result in a better filtration. In addition to apoptosis, cisplatin also induces tubular cell necrosis, especially in the proximal compartment [4], which is a strong pro-inflammatory stimulus. Cellular necrosis and tissue destruction activate an inflammatory response that, in these pathological circumstances, appears to amplify tissue injury, which further exacerbates inflammation [29, 46]. Both in the primary cell and tissue destruction and the secondary inflammatory response, oxidative stress seem to play a significant role. As such, amelioration of redox status by quercetin very probably contributes to tubular protection. *In vitro* studies have confirmed the cytoprotective efficacy of quercetin in endothelial [47] and glomerular cell lines [48], apparently through its antioxidant properties. Cisplatin promotes increased production of ROS, which can lead directly or indirectly, through several redox-sensitive signalling pathways, to necrosis and apoptosis of tubular epithelial cells [9, 45, 49], followed by an increased expression of pro-inflammatory mediators that intensify the cytotoxic effect [29].

Oxidative stress is also involved in the inflammatory response [50]. ROS can induce inflammatory processes via activation of transcription factors such as NF-κB, which in turn induce the production of pro-inflammatory cytokines, such as TNF-α. As such, the beneficial effect of quercetin might also be related to amelioration of inflammation. In fact, quercetin is able to suppress lipopolysaccharide-induced TNF-α and NO production through attenuation of NF-κB activity in macrophages, microglia cells and mast cells [51]. Tribolo *et al.* [52] reported that both quercetin and its metabolites, at physiological concentrations, can inhibit the expression of ICAM-1 and VCAM-1, two key molecules involved in monocyte recruitment during the early stages of inflammation, in activated human vascular endothelial cells. Francescato *et al.* [29] showed that quercetin exerts a protective effect on cisplatin-induced acute tubular necrosis in rats, which was associated to a decrease in the immunostaining for NF-κB and ED1 (a marker for macrophages). In addition, previous studies conduced in our laboratory demonstrated that quercetin prevented cadmium-induced nephrotoxicity in rats through an attenuation of iNOS and COX-2 overexpression, two key enzymes involved in the inflammatory process [17].

Besides tubular protection, direct haemodynamic effects may also be invoked to explain the effect of quercetin in the maintenance of GFR through the preservation of blood supply to the kidney. It has been reported that quercetin and its methylated metabolite isorhamnetin exhibit endothelium-independent vasodilator effects *in vitro* [53, 54]. In our study, a plausible explanation for the haemodynamic effects of quercetin is a reduction of RVR via either (i) a direct ROS scavenger effect and, particularly, to a diminished superoxide-driven NO inactivation [55] or (ii) potentiating the bioavailability and the biological activity of nitric oxide (NO) as a major regulatory factor of renal haemodynamics [56, 57].

The safety of quercetin is another advantage for its potential introduction as a renoprotective agent. Pharmacokinetic studies in humans have demonstrated that a once daily regime of quercetin (700–1000 mg/day or 8–10 mg/kg/day) can be safely administered to healthy volunteers or patients suffering from diseases related to oxidative stress, such as hypertension [58]. In animal studies, very high-dose quercetin (2000 mg/Kg) was reasonably safe for long-term administration in rats [59].

These results reinforce the prospective utility of quercetin in clinical practice as an adjuvant in antineoplastic therapies involving platinated molecules such as cisplatin. Indeed, co-administration of quercetin clearly improves the pharmacotoxicological profile of this family of drugs by reducing their most important side

effect. Specifically, protective adjuvants may be potentially used to (i) increase the dosage of cisplatin in patients who would benefit from it but did not hitherto qualify for it based on the risk of deadly nephrotoxicity and (ii) prevent the tolerable degree of nephrotoxicity occurring in a subset of patients treated with standard regimes of platinated drugs.

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