Original Article

Protective Effects of Quercetin on Cadmium-induced Cytotoxicity in Primary Cultures of Rat Proximal Tubular Cells*

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Abstract

Objective To investigate the protective effects of quercetin on cadmium-induced cytotoxicity in primary cultures of rat proximal tubular (rPT) cells.

Methods Primary cultures of rPT cells undergoing exponential growth were incubated with 1.0 μg/mL quercetin and/or cadmium (2.5, 5.0 μmol/L), in a serum-free medium at 37 °C at different time intervals. Commercial kits were used and flow cytometric analyses were performed on rPT cell cultures to assay apoptosis and oxidative stress.

Results Exposure of rPT cells to cadmium acetate (2.5, 5.0 μmol/L) induced a decrease in cell viability, caused an increase in apoptotic rate and apoptotic morphological changes. Simultaneously, elevation of intracellular reactive oxygen species, malondialdehyde and calcium levels, depletion of mitochondrial membrane potential and intracellular glutathione, and inhibition of Na^+, K^+-ATPase, Ca^{2+}-ATPase, glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) activities were revealed during the cadmium exposure of rPT cells. However, simultaneous supplementation with 1 μg/mL quercetin protected rPT cells against cadmium-induced cytotoxicity through inhibiting apoptosis, attenuating lipid peroxidation, renewing mitochondrial function and elevating the intracellular antioxidants (non-enzymatic and enzymic) levels.

Conclusion The present study has suggested that quercetin, as a widely distributed dietary antioxidant, contributes potentially to prevent cadmium-induced cytotoxicity in rPT cells.

Key words: Cadmium; Quercetin; Oxidative stress; Apoptosis; Proximal tubular cells; Primary cell culture

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INTRODUCTION

Cadmium (Cd) is known to be an occupational hazard and an environmental pollutant. Unlike complex organic pollutants, cadmium cannot be degraded by microorganisms. Instead, it accumulates in ecosystems and enters the food chain through environmental contamination of soil and water for an extremely long biological half-life. Long-term exposure to cadmium results in direct toxic effects[1]. It exerts multiorgan toxic effects in mammals and the kidney is the critical target organ for cadmium toxicity. The nephrotoxicity of cadmium

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has been extensively studied and widely reported in literatures. There is growing evidence that oxidative stress via reactive oxygen species (ROS) generation and mitochondrial damage are among the fundamental molecular mechanisms of cadmium nephrotoxicity[2-4]. Moreover, apoptotic death formed in the presence of cadmium could be responsible for its toxic effects[3,5-7].

In recent years, numerous studies have been exploring the therapeutic properties of extracts from different parts of various medicinal plants. The positive effects of flavonoids on human health have been given much attention. Flavonoids are natural phenolic substances that act as antioxidants in biological systems[8]. Quercetin, one of the most abundant flavonoids, is widely distributed in fruits and vegetables like apples, onion, mulberry, potatoes, broccoli, tea, peanuts, soybeans and red wine and is a potent oxygen free radicals scavenger[8-9]. It has been shown that quercetin has highly potent antioxidant and cytoprotective effects in preventing apoptosis and oxidative damage induced by cadmium in both *in vivo* and *in vitro* studies[2,7]. In view of these considerations, quercetin was chosen to evaluate its protective effect on cadmium-induced cytotoxicity in this study.

However, concerning the protective effect of quercetin on Cd-induced nephrotoxicity, only some experiments concentrated on *in vivo* studies and primary cultures of tubular cells were minimally applied[2,8]. Cell cultures are valuable tools for mechanistic studies at the cellular level. Besides, primary cultures can better represent the live tissue than cultures of permanent cell lines, and are therefore ideal for *in vitro* toxicity studies. Moreover, the renal tubule is one of major sites of renal impairment[10]. Hence, a very pure preparation of isolated proximal tubular cells from rat kidneys was used for culture in this study. In our previous studies, it has been confirmed that apoptotic cell death mediated by oxidative stress plays a key role in renal damage induced by cadmium exposure *in vitro* at lower exposure levels[6]. Therefore, a series of indices related to oxidative stress and apoptosis were chosen to elucidate the protective effect of quercetin on cadmium-induced cytotoxicity in primary cultures of rPT cells in order to provide a protective means against environmental Cd-induced renal damage and generate more comprehensive and reliable data for toxicological risk evaluation.

MATERIALS AND METHODS

Chemicals

All chemicals were of highest grade purity available. Cell Counting Kit-8 was obtained from Dojindo Laboratories (Tokyo, Japan) and Annexin V-FITC Apoptosis Detection kit from Pharmingen (Becton Dickinson Company, USA). E-cadherin (L-CAM) was purchased from BD Transduction Laboratories (Lexington, KY). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Material (Hangzhou, China). Malondialdehyde (MDA) assay kit, glutathione peroxidase (GSH-Px) assay kit, Na^+, K^+-ATPase assay kit and Ca^{2+}-ATPase analysis kit were from Keygen Biotech, Co., Ltd. (Nanjing, Jiangsu, China). N, N, N', N'-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) and Fluo-3/AM were purchased from Molecular Probes, USA. DMEM-F_{12} (1:1), cadmium acetate ($CdAc_2$), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT), propidium iodide (PI), 5'-chloromethylfluorescein diacetate (CMF-DA), 2', 7'-dichlorofluorescein diacetate (DCFH-DA), Hoechst 33258 staining, antibiotic-antimycotic solution, rhodamine 123 (Rh 123), collagenase, trypsin, quercetin (purity>98%), EDTA/EGTA and all other chemicals were purchased from Sigma-Aldrich, USA.

Cell Isolation, Cell Culture, and Exposure Conditions

Isolation of rPT cells has been described in our previous study[11]. The primary cells reached confluence after 144 h of cultivation and were subcultured after a 6-day culture by the Trypsin-EDTA digestive method. Both primary cells and subcultures were cultured in DMEM/F_{12} medium supplemented with 25 mmol/L HEPES, 10% FBS (heat-inactivated at 56 °C), 0.25 g/L of glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37 °C in the presence of 95% air and 5% CO_2. Identity of rPT cells was confirmed by staining with antibodies against specific proximal tubular antigens (immunocytochemical staining). Staining with E-cadherin (L-CAM) antibody was carried out to confirm the absence of other types of kidney cells, such as distal tubular cells, collecting duct cells and glomerulum-derived cells[12]. Cell purity of the isolated rPT cells was routinely higher than 95%. The first passage was used to perform the experimental design when cultured for 120 h, which was in its highest cell viability (according to the

growth curve, data not shown). Based on the doses of Cd in the previous studies, 2.5 μmol/L Cd and 5.0 μmol/L Cd were applied in this study[6]. Regarding the optimal dose of quercetin chosen for this study, cells were treated with a range of quercetin doses (0, 0.1, 1.0, 10 μg/mL) and/or 5.0 μmol/L Cd for 12 h, and cell viabilities were tested using MTT assay. The stock solution of $CdAc_2$ and quercetin was dissolved in sterile ultrapure water and ethanol, respectively. The concentration of ethanol in the medium was ≤0.1%. Based on an initial screening, cell cultures undergoing exponential growth were incubated with 1.0 μg/mL quercetin and/or Cd (2.5, 5.0 μmol/L), in a serum-free medium at 37 °C at different time intervals to detect a series of indices. Six groups were set up which were A: control; B: 1 μg/mL quercetin; C: 2.5 μmol/L Cd; D: 2.5 μmol/L Cd + 1 μg/mL quercetin; E: 5.0 μmol/L Cd; F: 5.0 μmol/L Cd + 1 μg/mL quercetin. Primarily, events over a 12-h exposure period were chosen for specific study.

Cell Viability Assay

Cell Counting Kit-8 is a one-bottle solution, which contains water-soluble tetrazolium salt. It can reduce the dehydrogenase in the mitochondria to water-soluble formazan dyes. The absorbance of these formazan dyes at 450 nm is proportional to the number of viable cells in the medium. Cells were seeded at a density of 1×10^4 in 96-well plates. After the preprocessing, the cells were treated with a series of Cd and/or quercetin concentrations for 12 and 24 h to assess the cytoprotective effect of quercetin on cell survival. At the destined time points, cell viability assays were performed using Cell Counting Kit-8, according to manufacturer's instructions. The absorbance was read at 450 nm by the microplate reader (Sunrise, Austria).

Hoechst 33258 Staining

Morphology of apoptotic cell nuclei was detected by staining with the DNA binding fluorochrome Hoechst 33258 (bisbenzimide). rPT cells were seeded on sterile cover glasses placed in the 24-well plates. After incubation with various quercetin and/or Cd concentrations for 12 h, cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min, and then incubated with Hoechst 33258 staining solution (50 μmol/L in PBS) for 10 min in the dark at room temperature. After three washes with PBS, the cells were viewed under an inverted fluorescence microscope (Olympus, IX-70, Japan) at an excitation wavelength of 352 nm through FITC filter (blue fluorescence). For assessing the extent of the apoptosis induced by quercetin and/or Cd, 200 cells were randomly selected to count those apoptotic cells within every batch of experiment, and each one was performed in triplicate.

Flow Cytometric Analysis

All the following assays were carried out on BD-FACS Aria flow cytometer and the 488 nm laser was utilized. Cells were seeded at a density of 3×10^5 in 6-well plates and treated with various quercetin and/or Cd concentrations for 12 h. Afterward, the adherent cells were collected with trypsin-EDTA solution (1.25 g/L trypsin, 0.2 g/L EDTA). The detached and adherent cells were pooled and harvested by centrifugation at 1500 rpm for 5 min. The harvested cells (at least 1×10^6 cells per group) were washed twice with PBS and incubated with various fluorescent dyes for the flow cytometric analysis. Cell debris, characterized by a low FSC/SSC were excluded from analysis. The data were analyzed by Cell Quest software and mean fluorescence intensity was obtained by histogram statistics.

Quantitation of Apoptosis Detection of apoptotic cell distribution was performed using annexin V/PI staining assay. The amount of early apoptotic cells, late apoptotic cells, living cells and necrotic cells was determined as the percentage of annexin V^+/PI^-, annexin V^+/PI^+, annexin V^-/PI^-, and annexin V^-/PI^+ cells, respectively[13]. The total apoptotic proportion therefore included the percentage of cells with fluorescence annexin V^+/PI^- and annexin V^+/PI^+. After 12 h exposure, the harvested cells were labeled with annexin V-FITC and PI using an apoptosis detection kit according to the manufacturer's protocol. The FITC and PI fluorescence was measured through FL-1 filter (530 nm) and FL-2 filter (585 nm), respectively, and 10 000 cells were analyzed.

Reactive Oxygen Species (ROS) Measurement Generation of ROS was monitored by measurement of hydrogen peroxide (H_2O_2) generation using the fluorescent probe DCFH-DA. This dye is cleaved to form non-fluorescent dichlorofluorescein (DCFH) in the cells, which gets oxidized to fluorescent dichlorofluorescein (DCF) by ROS. The DCF fluorescence intensity is proportional to the amount of peroxides produced by the cells. Briefly, 1.5×10^6 harvested cells/mL was incubated with DCFH-DA (100 μmol/L final concentration) for 30 min in the dark at 37 °C. The incubated cells were harvested

and suspended in PBS and ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 10 000 cells on flow cytometer.

Mitochondrial Membrane Potential (ΔΨ) Assay For the detection of mitochondrial ΔΨ, the harvested cells were incubated with Rh 123 (5 μg/mL final concentration) for 30 min in the dark at 37 °C, harvested and resuspended in PBS. The mitochondrial ΔΨ was measured by the fluorescence intensity (FL-1, 530 nm) of 10 000 cells.

Analysis of Intracellular Free Ca^{2+} Concentration ($[Ca^{2+}]i$) Following the treatment, the harvested cells were incubated with 0.5 mmol/L of TPEN (cell-permeate heavy metal chelator, which has high affinity to Cd^{2+} and low affinity to Ca^{2+}) to discount Cd interference in the fluorescence signal, because Ca^{2+} and Cd^{2+} interacted in a similar way[14]. Then, the cells were loaded with Fluo-3/AM (1 μmol/L final concentration) for 30 min in the dark at 37 °C, and washed with D-Hank's balanced salt solution afterwards (Ca^{2+}-free and Mg^{2+}-free). Intracellular $[Ca^{2+}]i$ levels were represented with fluorescent intensity (FL-1, 530 nm) of 10 000 cells on flow cytometer.

Glutathione (GSH) Measurement The cellular level of GSH was monitored by CMF-DA. This fluorescent probe reacts with intracellular sulfhydryls and reflects predominantly GSH[15]. The harvested cells were incubated with CMF-DA (1 μmol/L final concentration) for 30 min in the dark at 37 °C. Then, the incubated cells were harvested, suspended in PBS, and GSH was measured by the fluorescence intensity (FL-1, 530 nm) of 10 000 cells[16].

Determination of Other Indices

Cells were seeded at a density of $(6\text{-}8)\times10^5$ in 4-well plates, treated with various quercetin and/or Cd concentrations for 12 h. After the treatment, the detached and adherent cells were pooled and harvested by centrifugation to measure the following assays.

Measurement of Intracellular Lipid Peroxides (MDA) MDA is a breakdown product of the oxidative degradation of cell membrane lipids and is generally considered as an indicator of lipid peroxidation. The harvested cells were lysed in ice-cold physiological saline by sonication followed by centrifugation at 13 000 rpm for 5 min at 4 °C. The resulting supernatants were then used immediately for measuring the levels of MDA. The quantification was based on measuring formation of thiobarbituric acid (TBA) reactive substances (TBARS) according to the manufacturer's protocol. TBA was added to each sample tube and vortexed. The reaction mixture was incubated at 95 °C for 40 min. After cooling, the chromogen was read spectrophotometrically at 532 nm. The protein concentrations of the samples were determined by the method of Lorry et al.[17] to normalize the levels of MDA. And MDA levels were expressed in nmol/mg protein.

Activities of Na^+, K^+-ATPase, and Ca^{2+}-ATPase The harvested cells were homogenized in ice-cold physiological saline in an ultrasonic disintegrator. The cell homogenates were then centrifuged at 13 000 rpm for 10 min, and supernatants were obtained, whose protein concentration was determined by the Folin phenol method, using bovine serum albumin as a standard (the protein concentration in the assay was 3-5 mg /mL)[17]. The ATPases activities were assayed by the quantization of phosphonium ions, which were performed in accordance with the ATPase detection protocol.

Measurement of Glutathione Peroxidase (GSH-Px) Activity The harvested cells were lysed in ice-cold physiological saline by sonication followed by centrifugation at 13 000 rpm for 5 min at 4 °C. The resulting supernatants were then used immediately for measuring the activity of GSH-Px. The activity of GSH-Px was assessed according to the kit's instruction which makes use of the reaction: $H_2O_2+2GSH\rightarrow2H_2O+GSSG$ (oxidized glutathione). The absorbance was determined at 412 nm and the enzyme activity was expressed as μmol/mg protein.

Measurement of Catalase (CAT) Activity After the treatment, 50 mmol/L of potassium phosphate (pH 7.2) was added to the collected cells, and then the samples were sonicated. The cellular suspension was centrifuged in an Eppendorf tube at 13 000 rpm for 4 min and at 4 °C, and CAT activity was measured in the supernatant[18]. Results were expressed as increment of optical density per minute per milligram protein (ΔOD/min/mg.prot).

Superoxide Dismutase (SOD) Activity After the treatment, 50 mmol/L Tris-KCl (pH 8.2) was added to the collected cells, and then sonicated. The cellular suspension was centrifuged at 13 000 rpm for 4 min at 4 °C. SOD activity was measured in supernatants according to Marklund and Marklund[19].

Statistical Analysis

Data from the present study are presented as mean±SD from at least three independent experiments with different batches of cells, and each one was performed in duplicate or triplicate.

Statistical comparisons were made using one-way analysis of variance (ANOVA) (Scheffe's F test) after ascertaining the homogeneity of variance between the treatments. All statistical data were analyzed using SPSS 13.0 (SPSS, Chicago, IL, USA). The critical value for statistical significance was $P<0.05$.

RESULTS

Protective Effect of Quercetin on Cellular Death of rPT cell Induced by Cadmium

Compared with the control, rPT cell viability manifested no significant change after treatment with 0.1-1.0 μg/mL quercetin alone for 12 h ($P>0.05$). However, rPT cell treated with 10 μg/mL quercetin alone resulted in a decrease in cell viability ($P>0.05$; Table 1). Also, incubation of rPT cell with 1.0 μg/mL quercetin exhibited the highest inhibition of cellular death against 5.0 μmol/L Cd. Thus, 1.0 μg/mL quercetin was chosen to evaluate its protective effect on cellular death induced by Cd exposure. As shown in Table 2, the rPT cells were incubated with 1.0 μg/mL quercetin in the presence of Cd (2.5, 5.0 μmol/L) for 12 and 24 h, and as a result , the cellular death induced by cadmium could be significantly reversed by quercetin ($P<0.01$).

Effect of Quercetin on Cadmium-induced rPT cell Apoptosis

The apoptosis of rPT cell induced by quercetin and/or Cd was performed by flow cytometric analysis (Figure 1). After 12 h exposure, the number of apoptotic cells enhanced significantly ($P<0.01$), being 4.55-fold and 6.28-fold of that of the control after exposure to 2.5 μmol/L Cd and 5.0 μmol/L Cd, respectively; while the apoptosis was significantly prevented by quercetin ($P<0.01$), being 2.09-fold and 3.66-fold of that of the control after exposure to (1.0 μg/mL quercetin + 2.5 μmol/L Cd) and (1.0 μg/mL quercetin + 5.0 μmol/L Cd), respectively. However, quercetin treatment alone did not affect apoptosis ($P>0.05$).

Effect of Quercetin on Cadmium-induced Apoptotic Morphological Changes of rTP Cells

Apoptotic morphological changes in the nuclear chromatin of rTP cells were detected by staining with the DNA binding fluorochrome Hoechst 33258 (Figure 2). In the control and quercetin groups, the majority of cells appeared normal with uniformly stained nuclei and the chromatin of normal nuclei was unaltered and spread uniformly throughout the entire nucleus. Whereas Cd-treated cells exhibited morphological changes of typical apoptosis, fragmented chromatin was characterized by a scattered, drop-like structure and condensed chromatin was located at the periphery of the nuclear membrane and appeared as a half-moon form. The nuclei of apoptotic cells appeared smaller and shrunken compared with intact cells. However, combined treatment with 1.0 μg/mL quercetin restored all these parameters to similar levels in comparison with the control (Figure 2). In addition, the statistical result of cells with apoptotic characteristics showed that the number of apoptotic cells were enhanced progressively with the cadmium concentration when they were exposed to cadmium alone. Regarding the extent of the apoptosis, the combined groups [(1.0 μg/mL quercetin + 2.5 μmol/L Cd), (1.0 μg/mL quercetin + 5.0 μmol/L Cd)] showed less lesion ($P<0.01$) than the group treated with cadmium alone (2.5, 5.0 μmol/L Cd).

Table 1. Effect of Different Doses of Quercetin on rPT Cell Viabilities after 12 h Exposure ($n=6$)

$CdAc_2$	Concentrations of Quercetin (μg/mL)			
	0	0.1	1.0	10
0	100.0±2.98^{a}	100.3±3.02^{a}	101.6±3.22^{a}	97.96±2.92^{a}
5 μmol/L Cd	84.48±2.76^{b}	88.01±2.81^{b}	93.35±2.49^{c}	89.92±2.63^{cd}

Note. Values with different superscripts are statistically different ($P<0.05$).

Table 2. Protective Effect of Quercetin on Cellular Death of rPT Cell Induced by Cadmium ($n=6$)

Group	12 h	24 h
A	100.00±3.02	100.00±3.81
B	99.62±2.92^{ns}	99.80±2.88^{ns}
C	91.18±3.32$^{\#}$	84.98±3.99$^{\#}$
D	97.41±2.76*	93.92±3.88**
E	85.36±3.21$^{\#}$	75.09±4.82$^{\#}$
F	92.66±3.17**	85.84±4.60**

Note. Cell viabilities of the control group at the destined time points were assumed as 100%, and the variations were represented as percentage. $^{\#}$Statistical significance between control (A) and single Cd treatment (C, E); *Statistical significance between cells cultured in the absence (A, C, E) and presence (B, D, F) of quercetin. ns Not significant; $^{\#}P<0.01$; $^{*}P<0.05$, $^{**}P<0.01$.

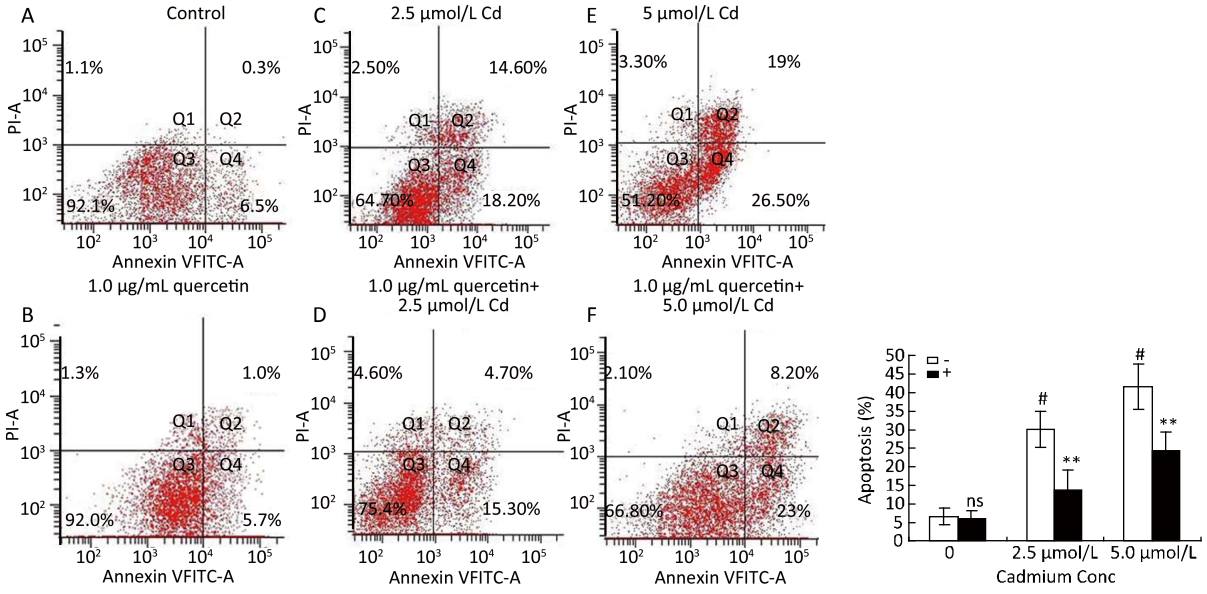

Figure 1. Effect of quercetin on apoptosis induced by Cd in rPT cells. Results are mean±SD of three separate experiments with each one performed in triplicate (*n*=9).Two different colors were chosen to point out which cells were treated with quercetin (+) and which were not (-); i.e., white columns for no quercetin-treatment and black for quercetin-treatment. #Statistical significance between control and Cd treatment among no quercetin-treated groups; *Statistical significance between cells cultured in the absence (-) and presence (+) of quercetin. ns Not significant; #*P*<0.01;** *P*<0.01.

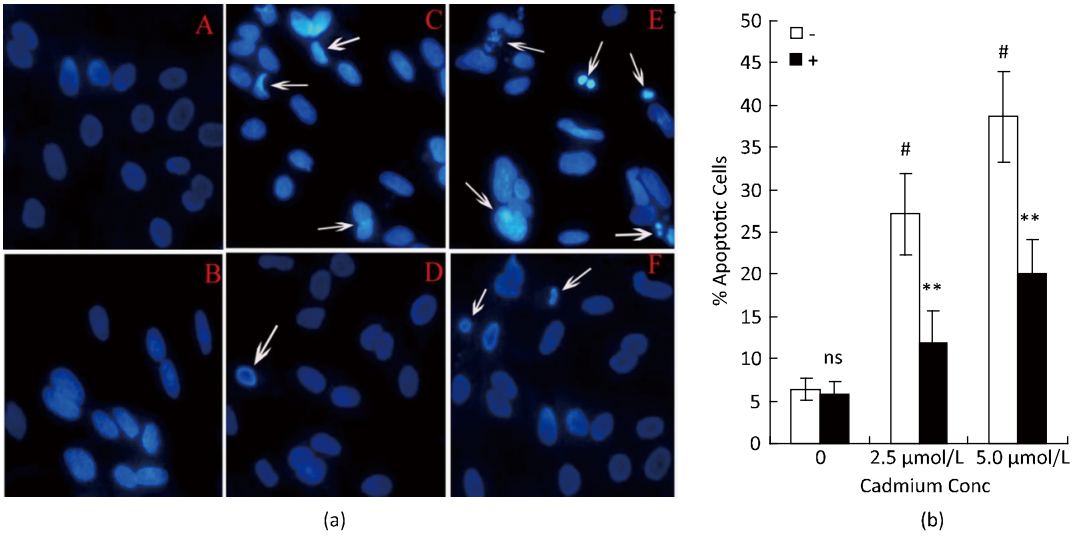

Figure 2. Effects of Cd and/or quercetin on apoptotic morphological changes in rPT cells. (a) Changes of nuclei fragmentation with condensed chromatin are evident (thin arrows). A-F: original magnification ×400. (b) Results are mean±SD of three separate experiments and each one is performed in triplicate (*n*=9). Two different colors were chosen to point out which cells were treated with quercetin (+) and which were not (-); i.e., white columns for no quercetin-treatment and black for quercetin-treatment. #Statistical significance between control and Cd treatment among no quercetin-treated groups; *Statistical significance between cells cultured in the absence (-) and presence (+) of quercetin. ns Not significant; #*P*<0.01;** *P*<0.01.

Effect of Quercetin on Cadmium-induced ROS Generation and Intracellular MDA Levels

The generation of intracellular ROS was detected by using the fluorescent probe DCFH-DA. As shown in Table 3, the level of ROS was increased by the treatment with single cadmium in a dose-dependent manner, being 2.94-fold and 3.68-fold of that of the control after exposure to 2.5 μmol/L Cd and 5 μmol/L Cd, respectively. Exposure to the combined groups led to a significant (P<0.01) decrease in ROS generation compared with the single Cd exposure, being 1.38-fold and 2.32-fold of that of the control after exposure to (1.0 μg/mL quercetin + 2.5 μmol/L Cd) and (1.0 μg/mL quercetin + 5.0 μmol/L Cd), respectively. Moreover, the level of intracellular MDA (a common end product of lipid peroxidation) was measured to study the protective effect of quercetin on Cd-induced oxidative lesions. The dose-dependent generation of MDA levels increased significantly (P<0.01) following single cadmium exposure, compared with the control group. When 1.0 μg/mL quercetin was co-administered with Cd for 12 h, Cd-induced intracellular MDA levels were shown to have been significantly attenuated by quercetin.

Table 3. Effects of Cd and/or Quercetin on Intracellular MDA Levels, Generation of ROS and Mitochondrial ΔΨ in rPT Cells (n=6)

Group	MDA (nmol/mg. prot)	Intracellular ROS (MFI)	Mitochondrial ΔΨ (MFI)
A	0.1256±0.0321	8668±1672	30908±1672
B	0.1129 ± 0.0412^{ns}	8501 ± 1908^{ns}	31412 ± 1908^{ns}
C	$0.3266\pm0.0792^{\#}$	$25456\pm3200^{\#}$	$24222\pm1821^{\#}$
D	$0.1628\pm0.0592^{**}$	$12001\pm3521^{**}$	$28146\pm1666^{**}$
E	$0.5233\pm0.1016^{\#}$	$31908\pm4322^{\#}$	$19820\pm2568^{\#}$
F	$0.2809\pm0.0924^{**}$	$20103\pm4490^{**}$	$25622\pm2302^{**}$

Note. Intracellular ROS levels and mitochondrial ΔΨ were expressed as mean fluorescence intensity (MFI). $^{\#}$Statistical significance between control (A) and single Cd treatment (C, E); *Statistical significance between cells cultured in the absence (A, C, E) and presence (B, D, F) of quercetin. ns Not significant; $^{\#}P$<0.01; $^{**}P$<0.01.

Effect of Quercetin on Cadmium-induced Mitochondrial Membrane Potential (ΔΨ)

Rh 123, a lipophilic cationic fluorescent dye, is selectively taken up by mitochondria and is used therefore to analyze changes in mitochondrial ΔΨ during the apoptotic cell death. As shown in Table 3, a significant decrease in mitochondrial ΔΨ occurred in rPT cells after exposure to cadmium (2.5, 5.0 μmol/L) for 12 h (P<0.01). Likewise, 1.0 μg/mL quercetin exhibited a significant (P<0.01) increase in the loss of mitochondrial ΔΨ induced by cadmium exposure. However, quercetin treatment alone had no effect on mitochondrial ΔΨ (P>0.05).

Effects of Quercetin and/or Cadmium on Intracellular $[Ca^{2+}]i$ and Activities of Na^+, K^+-ATPase and Ca^{2+}-ATPase

After incubation with $CdAc_2$ (2.5, 5.0 μmol/L) for 12 h, marked enhancement of intracellular $[Ca^{2+}]i$ (P<0.01) and significant inhibition of Na^+, K^+-ATPase activity and Ca^{2+}-ATPase activity (P<0.01) were observed in a dose-dependent manner, as compared with the control group (see Table 4). Furthermore, intracellular calcium overload and loss in Na^+, K^+-ATPase and Ca^{2+}-ATPase activities induced by Cd exposure could be significantly reversed by 1.0 μg/mL quercetin (P<0.01). However, quercetin treatment alone had no effect on these assays (P>0.05).

Table 4. Effects of Cd and/or Quercetin on $[Ca^{2+}]I$ and ATPases Activities in rPT Cells (n=6)

Group	Intracellular $[Ca^{2+}]i$ (MFI)	Activities of Na^+, K^+-ATPase (μmol Pi/mg protein/h)	Activities of Ca^{2+}-ATPase (μmol Pi/mg protein/h)
A	682±58	2.42±0.220	2.12±0.128
B	661 ± 64^{ns}	2.55 ± 0.310^{ns}	2.20 ± 0.197^{ns}
C	$992\pm106^{\#}$	$1.84\pm0.201^{\#}$	$1.43\pm0.173^{\#}$
D	$743\pm91^{**}$	$2.28\pm0.195^{**}$	$2.01\pm0.202^{**}$
E	$1284\pm110^{\#}$	$1.30\pm0.119^{\#}$	$1.16\pm0.141^{\#}$
F	$901\pm115^{**}$	$1.89\pm0.211^{**}$	$1.81\pm0.211^{**}$

Note. Intracellular calcium levels were expressed as mean fluorescence intensity (MFI). $^{\#}$Statistical significance between control (A) and single Cd treatment (C, E); *Statistical significance between cells cultured in the absence (A, C, E) and presence (B, D, F) of quercetin. ns Not significant; $^{\#}P$<0.01; $^{**}P$<0.01.

Effect of Quercetin on Cadmium-induced Changes in Antioxidant Status of rTP Cells

The GSH level and GSH-Px, CAT, and SOD activities of rTP cells were determined to reflect intracellular antioxidant status. After incubation with

$CdAc_2$ (2.5, 5.0 μmol/L) for 12 h, a significant (P<0.01) decrease in the level of nonenzymic antioxidant (GSH) and significantly decreased activities of enzymic antioxidants (GSH-Px, CAT, and SOD) (P<0.01) were observed, as compared with the control group (Table 5). The reduction of GSH levels and these antioxidases activities were in a dose-dependent manner. However, compared with single cadmium exposure (2.5, 5.0 μmol/L), the GSH level and GSH-Px, CAT, and SOD activities displayed a significant increase in the presence of 1.0 μg/mL quercetin (P<0.01), or, quercetin supplementation (1.0 μg/mL) significantly (P<0.01) restored the intracellular antioxidant levels in rPT cells. In contrast, rPT cells treated with quercetin alone did not show significant changes (P>0.05) in the GSH level and activities of GSH-Px, CAT, and SOD when compared with control values.

Table 5. Descriptive Statistics of Oxidative Stress in Different Treatment Groups (n=6)

Group	GSH (MFI)	GSH-Px (μmol/mg. prot)	CAT (ΔOD/min/mg prot)	SOD (ng/hr/mg protein)
A	1263±102	40.2±3.98	3.35±0.238	3601±209
B	1299±110^{ns}	42.1±3.06^{ns}	3.41±0.252^{ns}	3722±299^{ns}
C	961±83$^{\#}$	31.5±3.18$^{\#}$	2.54±0.302$^{\#}$	2820±301$^{\#}$
D	1201±110**	39.5±3.82**	3.29±0.248**	3502±204**
E	793±94$^{\#}$	26.9±4.02$^{\#}$	2.30±0.301$^{\#}$	2409±237$^{\#}$
F	1102±121**	37.5±2.47**	3.06±0.201**	3401±298**

Note. Intracellular GSH levels were expressed as mean fluorescence intensity (MFI). $^{\#}$Statistical significance between control (A) and single cadmium treatment (C, E) among the no quercetin-treated groups; *Statistical significance between cells cultured in the absence (A, C, E) and presence (B, D, F) of quercetin. ns Not significant; $^{\#}P$<0.01; $^{**}P$<0.01.

DISCUSSION

Cadmium is a potent nephrotoxic toxicant. There have been numerous investigations focused on the therapeutic effects of some extracts from medicinal plants on cadmium-induced nephrotoxicity. Quercetin is a widely distributed dietary antioxidant, showing potential of preventing cadmium induced nephrotoxicity *in vivo*[2,8,20]. However, little is known about the protective effect of quercetin on cadmium-induced cytotoxicity *in vitro*. Due to the fact that primary cultures are able to better represent the live tissue, they are ideal for *in vitro* toxicity studies. Moreover, the proximal tubule is a sensitive target site of renal damage induced by cadmium exposure[10]. Herein, this study was designed to investigate the intracellular events in primary cultures of rat proximal tubular cells in order to better understand the protective mechanism of quercetin on cadmium nephrotoxicity. In addition, 1 μg/mL quercetin was selected to be the optimal concentration used in this study through an initial screening (Table 1). Primarily, events of cadmium exposure over 12 h were chosen to study the protective mechanism.

As shown in Table 2, cadmium exposure markedly decreased the cell viability of rPT cells in a time- and dose-dependent manner, whereas the cell death was significantly prevented by quercetin. In addition, no evidence of toxicity of quercetin was shown at a dose of 1.0 μg/mL. Our previous studies[6] demonstrated that cell death in rPT cells induced by low-concentration of cadmium (2.5-5.0 μmol/L) exposure was mediated by two mechanisms, namely necrotic and apoptotic. Moreover, the apoptotic death played a key role in the cellular death induced by cadmium at these concentrations (2.5-5.0 μmol/L). It also appears that in the apoptotic death mediated by cadmium, rather than in the necrotic death, oxidative stress could be implicated[6]. Thus, indices related to oxidative stress and apoptosis were chosen for assessing the protective effect of antioxidant quercetin on apoptotic death induced by cadmium exposure in this study, while its protective effect on necrotic death induced by cadmium exposure was not discussed here, which we believe, will be worthy of further investigation.

Concerning the apoptotic rates measured by flow cytometry, the apoptosis induced by cadmium was prevented significantly by quercetin (Figure 1). Also, apoptotic morphological changes mediated by cadmium exposure, assessed by Hoechst 33258

staining, could be obviously reversed by simultaneous supplementation with antioxidant quercetin (Figure 2), which further indicated that the antagonistic effect of quercetin on apoptotic death was caused by cadmium in rPT cells. The changing tendency in the percentage of apoptotic cells (Figure 2) was consistent with the result obtained by flow cytometric analysis (Figure 1). However, quercetin alone caused no significant change in apoptotic rates and apoptotic morphological changes in rPT cells compared with the control, indicating that quercetin represented a low toxicity antioxidant and might play a role in attenuating the cytotoxicity caused by Cd in rPT cells.

Several studies confirmed that cadmium-induced nephrotoxicity was associated with oxidative stress that included lipid peroxidation and production of ROS[2,4,6,20-21]. Results from our study (Table 3) are in accordance with these reports, further demonstrating that oxidative stress played a critical role in the cytotoxicity induced by cadmium. However, simultaneous supplementation with antioxidant quercetin significantly alleviated the production of ROS as well as the enhanced generation of intracellular MDA levels. In this process attenuation of lipid peroxidation was a major action by quercetin. Moreover, it has been demonstrated that mitochondria play a crucial role in cadmium-induced apoptosis in rPT cells[6]. Mitochondria are the major source of intracellular ROS generation and, at the same time, an important target damaged by ROS[22]. Lipid peroxidation is involved in mitochondrial dysfunction and alterations in mitochondrial function are largely attributed to the damaging effects of ROS[23]. Also, large amounts of ROS may constitute a direct cause of mitochondrial dysfunction by inducing the mitochondrial permeability transition (mPT), and, as a result, lead to mitochondria swelling, loss of the mitochondrial ΔΨ, and further ROS production[24]. As shown in Table 3, cadmium exposure produced a breakdown of the mitochondrial ΔΨ in rPT cells, indicating that oxidative stress induced by cadmium may be due to mitochondrial dysfunction; while 1 μg/mL quercetin could renew mitochondrial dysfunction due to its ability to scavenge the intracellular ROS.

Previous studies suggest that intracellular calcium overload may be another important mechanism of the development of cadmium-induced apoptosis in rPT cells[6]. Intracellular Ca^{2+} levels are maintained by many factors. Among them, the Na^+, K^+-ATPase and Ca^{2+}-ATPase play critical roles in intracellular Ca^{2+} homeostasis, which act in the removal of Ca^{2+} from the cytosol across the plasma membrane, and blockage of these enzymes can induce the cytosolic calcium overload[25]. Inhibition of Na^+, K^+, and Ca^{2+}-ATPases activities by cadmium exposure in rPT cells (Table 4) may demonstrate this idea. In addition, Na^+, K^+, and Ca^{2+}-ATPases are adenosine triphosphate (ATP) dependent, indicating that sufficient ATP is necessary to maintain their function[26]. Since mitochondria are the major site of ATP production and mitochondrial ΔΨ is the driving force of ATP synthesis, a breakdown in the mitochondrial ΔΨ could lead to the fall in the ATP levels[27]. Therefore, the resultant deficiency in cellular ATP levels due to mitochondrial dysfunction leads to inhibition of the Na^+, K^+, and Ca^{2+}-ATPases[28-29]. Simultaneously, Na^+, K^+, and Ca^{2+}-ATPases are highly vulnerable to the effects of oxidative damage and free radical attack[29]. Therefore, it can be concluded that mitochondrial dysfunction induced by cadmium leading to the disruption of energy metabolism and overproduction of ROS (elevation of intracellular MDA levels) results in the decrease of ATPases activities, which are involved in intracellular calcium overload. In this study, quercetin abolished the Cd-stimulated elevation of intracellular free Ca^{2+} concentration and restored the cadmium-induced inhibition of Na^+, K^+, and Ca^{2+}-ATPases activities (Table 4), which may be concerned with its protective effect on mitochondrial function and potent efficacy in scavenging intracellular ROS. In other words, attenuation of intracellular calcium overload due to supplementation with quercetin will aid in inhibiting the Cd-induced apoptosis in rPT cells.

Quercetin is one of several dietary flavonoids that have been identified to occur naturally in fruits and vegetables at relatively high concentrations. Several studies have shown that quercetin is higly potent free radical scavenger via maintaining the intracellular redox balance[7,30-31]. In our study, we have observed that depletion of GSH levels, decreased activities of SOD, GPx, and CAT were seen in rPT cells exposed to cadmium (Table 5), further suggesting increased oxidative damage in these cells. However, simultaneous supplementation with antioxidant quercetin significantly alleviated the damaged antioxidant status to near the normal level of intracellular GSH, GSH-Px, CAT, and SOD activities (Table 5). These results suggest that quercetin represents a potent antioxidant that possesses

cytoprotective effects in elevating the non-enzymatic and enzymic antioxidants levels in rPT cells exposed to cadmium.

In summary, cadmium exposure induced cytotoxicity in primary cultures of rat proximal tubular cells involving mitochondrial oxidative damage with breakdown of mitochondrial ΔΨ, elevation of intracellular $[Ca^{2+}]i$, and ROS overproduction. These changes promoted the development of apoptosis. However, as a potent antioxidant, quercetin manifested a protective effect against cadmium-induced cytotoxicity in rPT cells through inhibiting apoptosis, attenuating lipid peroxidation, renewing mitochondrial function, followed by elevation of the non-enzymatic and enzymic antioxidants levels.

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