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Effects of apigenin on the expression levels of B-cell lymphoma-2, Fas and Fas ligand in renal ischemia-reperfusion injury in rats

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Abstract. The aim of the present study was to investigate the effect and possible mechanism of apigenin on renal ischemia-reperfusion (I/R) injury in rats, as well as in *in vitro* experiments. In total, 36 rats were subjected to 45 min of renal ischemia, with or without treatment prior to ischemia with different concentrations of apigenin (2, 10 and 50 mg/kg) administered intravenously. All rats were sacrificed at 24 h after I/R injury. The serum creatinine (Cr) and blood urea nitrogen (BUN) levels were analyzed, and histological examination was conducted. In addition, the expression levels of B-cell lymphoma 2 (Bcl-2) and Fas/Fas ligand (FasL) were detected by immunohistochemistry, reverse transcription-quantitative polymerase chain reaction and western blot analysis. For *in vitro* experiments, the NRK-52E cell line was employed. The viability, apoptosis and expression levels of Fas, FasL and Bcl-2 were examined in the culture of NRK-52E cells following the I/R. The results indicated that apigenin significantly decreased the levels of serum Cr and BUN induced by renal I/R, demonstrating an improvement in renal function. The histological evidence of renal damage associated with I/R was also mitigated by apigenin *in vivo*. Furthermore, apigenin increased the cell viability and decreased cell apoptosis in the culture of NRK52E after I/R *in vitro*. Compared with the I/R group, the expression of Bcl-2 was upregulated and the expression levels of Fas and FasL were downregulated by apigenin at the mRNA and protein levels *in vivo* and *in vitro*. In conclusion, apigenin appeared to increase the expression of Bcl-2 and reduce Fas/FasL expression in renal I/R injury, providing evident protection against renal I/R injury in rats.

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Key words: apigenin, ischemia, reperfusion, B-cell lymphoma 2, Fas/Fas ligand

Introduction

Renal ischemia/reperfusion (I/R) injury is a primary cause of acute renal failure, which is commonly observed in a number of clinical settings, including shock and renal transplantation (1,2). In addition, renal I/R injury is a leading contributor to the morbidity and mortality of the aging population, and results from the process of recovering the blood or oxygen supply following ischemia in the kidney. This process may then lead to cellular damage in renal tissues (3-5). Under these conditions, the tissue structure and renal function deteriorate, and tubular necrosis, medullary hemorrhage and congestion are observed. Patients with I/R injury have a poor prognosis and there is currently no available effective therapy to mitigate this injury (6).

Apigenin is a plant flavone that exist in a variety of fruits and vegetables, such as celery, parsley and wheat sprouts (7,8). This compound has been proven to possess a number of biological properties, including anti-inflammatory, antioxidant and antitumor effects (9), as well as a protective effect on several organs. Apigenin can protect cells against apoptosis and necrosis by inhibiting oxidative stress. Furthermore, it has been demonstrated that apigenin has a potent therapeutic effect on liver in rats (10). However, the effects of apigenin on renal I/R injury remain unclear.

In the kidney, the cellular death receptors Fas and Fas ligand (FasL) are major mediators of the apoptotic pathway. Fas belongs to the tumor necrosis factor (TNF) receptor superfamily of cell surface death receptors, and FasL is a member of the TNF family that induces apoptosis by cross-linking its Fas receptor (11,12). Several studies revealed that Fas/FasL are involved in multifarious forms of renal injury, including I/R injury and tubular injury in glomerulonephritis (13,14). Furthermore, the role of B-cell lymphoma 2 (Bcl-2) in the development of apoptotic cell death has been widely investigated (15,16). The Bcl-2 family of proteins regulates cell apoptosis and cell necrosis (17). The Fas/FasL and Bcl-2 genes are two of the key factors influencing apoptosis and regulating the intrinsic apoptosis pathway in renal tubular epithelial cells (18,19). In addition, the intrinsic apoptosis pathway is activated by oxidative stress, which is mediated by increased mitochondrial membrane permeability (20). Previous studies have suggested that oxidative stress is involved in the apoptotic mechanism mediated by the Fas/FasL signaling pathway (21-23). Therefore, it is important to verify whether

the renoprotective effect of apigenin are associated with the modulation of the Fas/FasL and Bcl-2 pathway.

The aim of the present study was to investigate the protective role of apigenin against renal I/R injury in rats and the underlying mechanism of its action. The study examined whether the protective effect of apigenin on renal function was through the modulation of the Fas/FasL pathway and improvement of the expression of Bcl-2.

Materials and methods

Ethical approval. The present study was approved by the Local Ethical Committee of the Renmin Hospital of Wuhan University (Wuhan, China), and the experimental procedures were performed in accordance with the principles of the Declaration of Helsinki.

Animals and in vivo experimental protocol. A total of 36 Sprague-Dawley rats (male; weight, 220±20 g; Hubei Provincial Academy of Preventive Medicine, Wuhan, China) were randomly separated into six groups (n=6 per group) based on the randomized block design method, as follows: i) Sham surgery; ii) I/R injury only; iii) apigenin (50 mg/kg) + sham; iv) I/R injury + 2 mg/kg apigenin treatment; v) I/R injury + 10 mg/kg apigenin treatment; and vi) I/R injury + 10 mg/kg apigenin treatment. In the three I/R + apigenin groups, apigenin was intravenously injected at 10 min prior to the induction of ischemia. Apigenin (purity, >98%) was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China).

All the rats were anesthetized with chloral hydrate intraperitoneally (350 mg/kg). After intravenous injection of heparin (1,000 UI/kg) and maintaining the body temperature at 37˚C, a midline laparotomy was performed. In the I/R groups, a right nephrectomy was performed, followed by isolation of the left renal pedicles (artery, vein and nerve). The left kidney was then subjected to 45 min of ischemia followed by reperfusion subsequent to right nephrectomy. During ischemia, the color of the kidneys changed to a purple shade, which was altered to a blush color during reperfusion. In the sham and apigenin + sham groups, rats were subjected to the same surgical procedures as the I/R groups without left renal clamping. At 24 h after I/R injury, all rats were sacrificed. Blood samples (1 ml) were collected from the heart for the measurement of serum creatinine (Cr) and blood urea nitrogen (BUN) levels. The left kidney was removed and fixed in 4% paraformaldehyde or immediately frozen, and stored at -80˚C for routine paraffin embedding and further examinations.

Serum assays. Blood samples were centrifuged at 15,000 x g for 10 min at 20˚C and the serum was stored at -20˚C until further analyses. Serum was analyzed according to the protocols of the Creatinine and Urea Assay kits (C013-1 and C011-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbance was measured using a spectrophotometer (UV-1700; Shimadzu Corporation, Tokyo, Japan) at 520 nm. Then, the concentrations of BUN and Cr were calculated.

Measurement of malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in renal tissues. The renal tissues were homogenized and centrifuged at 3,000 x g for 10 min at 4˚C. Then, the supernatants were collected for analysis of the MDA, SOD and GSH-Px activity using commercial SOD, MDA and GSH-Px assay kits (A001-1-1, A003-1 and A005; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

Histological examination of renal tissues. Half of each kidney was removed from the rats and fixed in 4% paraformaldehyde, followed by routine paraffin embedding. According to standard procedures, the tissues were cut into 4-μm sections and stained with hematoxylin and eosin (H&E) for histological grading. The sections were assessed by an experienced renal pathologist to determine the grading scores by Jablonski's standard (24) for the histopathological assessment of renal I/R injury.

Immunohistochemical analysis of renal tissues. The expression levels of Fas/FasL and Bcl-2 in renal tissues were examined by immunohistochemical staining. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide at 37˚C for 10 min. Next, the tissue sections were treated with 1:50 normal horse serum in Tris-buffered saline (TBS) for 30 min at 37˚C. Subsequently, rabbit anti-Bcl-2 (1:1,000 dilution; A2212; ABclonal, Woburn, MA, USA) or rabbit anti-Fas/FasL (1:500 dilution; A2639/A0234; ABclonal) antibodies were added to the tissues and incubated overnight at 4˚C. Phosphate-buffered saline (PBS) was then used for washing these sections three times. Subsequent to incubating with the anti-rabbit (1:100; sc-3753; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) secondary antibody for 30 min at 20˚C, the sections were treated with the DAB chromogen for visualization. The average optical density (AOD) was calculated from five random fields-of-view per slide using Image-Pro Plus software, version 5.0 (Media Cybernetics, Inc., Shanghai, China), and the AOD was presented as the mean value of three detections for each sample.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The extraction of total RNA from rat kidney tissues was performed using TRIzol RNA Reagent kit (Takara Bio, Inc., Otsu, Japan). RNA concentration was obtained by spectrophotometry. Single-stranded cDNA was synthesized using the cDNA synthesis kit (Takara Bio, Inc.) according to the manufacturer's protocol. Subsequently, qPCR was conducted with the Applied Biosystems SYBR-Green Mix kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction mixture contained: 2 μl cDNA, 12.5 μl 2X SYBR-Green mix, 1 μl forward primer, 1 μl reverse primer and 8.5 μl ddH 2 O, in a final volume of 25 μl. The primers used were as follows: Bcl-2 forward, 5'-TTTGATTTCTCCTGGCTGTCT-3' and reverse, 5'-CTGATTTGACCATTTGCCTG-3'; Fas forward, 5'-CAAGGGACTGATAGCATCTTTGAGG-3' and reverse, 5'-GTCCTTAACTTTTCGTTCACCCAGG-3'; FasL forward, 5'-TCCACCACCACCTCCATCAC-3' and reverse, 5'-CCA ACCTTACCCCAATCCTT-3'; GAPDH forward, 5-GGTCAT CAACGGGAAACCC-3' and reverse, 5'-TCTGAGTGGCAG TGATGGCA-3'. Analysis of the relative gene expression levels was performed using GAPDH as an endogenous reference gene. Bcl-2, Fas and FasL transcript levels were normalized to GAPDH transcript levels, with the mean values reported for each group.

Western blot assay. The kidney tissues were dissociated using a Total Protein Extraction kit (Wuhan Goodbio Technology Co., Ltd., Wuhan, China) according to the specifications of the kit. Total proteins extracted were then examined via western blot analysis. Briefly, 40 μg protein from each sample was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then blocked by 5% non-fat milk in TBS/Tween-20 (TBST) buffer and incubated with polyclonal primary antibodies of anti-Bcl-2 (1:500 dilution; A2212; ABclonal), anti-Fas/FasL (1:500 dilution; A2639/A0234; ABclonal) and anti-GAPDH (1:500 dilution; A10868; ABclonal) at 4˚C overnight. Subsequent to washing three times with TBST for ~15 min, the membranes were incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:200 dilution; sc-3753; Santa Cruz Biotechnology, Inc.). Membranes were then washed three times with TBST, and specific bands were visualized using an enhanced chemiluminescence detection kit (Immobilon Western Chemiluminescence HRP Substrate; Merck KGaA, Darmstadt, Germany). The band intensity was detected using the Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell culture for in vitro experiments. Rat renal tubular epithelial NRK-52E cells were purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 0.1 mg/ml streptomycin and 100 U/ml penicillin. The medium was replaced every 24 h.

In vitro experimental groups and treatments. The NRK-52E cells were randomly divided into six groups, as follows: i) Normal; ii) I/R only; iii) normal + 1,000 nM apigenin; iv) I/R + 10 nM apigenin; v) I/R + 100 nM apigenin; and vi) I/R + 1,000 nM apigenin. Prior to the experiment, all the groups were cultured for 24 h simultaneously. The normal group was incubated with a control medium (24.0 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 86.5 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 20 mM HEPES and 5 mM glucose; pH 7.4). The I/R group was cultured with an anoxia medium (4.5 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 106.0 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 20 mM morpholinoethanesulfonic acid; pH 6.6), and then exposed to hypoxia (0.5% O_2, 5% CO_2 and 94.5% N_2) at 37˚C for 3 h, followed by reoxygenation (21% O_2, 5% CO_2 and 74% N_2) at 37˚C for 24 h. Similarly, the I/R + apigenin (10, 100 and 1,000 nM) groups were subjected to the same procedure as the I/R group, but cells were pretreated for 24 h with apigenin prior to the procedure. In the normal + apigenin group, the same procedures were performed as for the normal group, along with pretreatment for 24 h with apigenin (1,000 nM).

Cell counting kit-8 (CCK8) assay. The NRK-52E cells were inoculated in a 96-well plate at a cell density of 10^5 cells/well. After 24 h of culture at 37˚C, the cells were treated with CCK8 solution, and the plate was incubated in an incubator for 4 h. The absorbance of cells at wavelength of 450 nm in terms of the OD was measured using a microplate reader. The cell survival rate was calculated according to the following formula: Survival (%) = (treatment group OD-blank control OD)/(normal group OD-blank control OD) x100%.

Annexin V-FITC/propidium iodide (PI) detection of apoptosis in NRK-52E cells. An Annexin V-FITC/PI assay was conducted using the Annexin V-FITC Apoptosis Detection kit (70-AP101-100; Liankebio, Hangzhou, China) according to the manufacturer's protocol, followed by flow cytometric analysis. Briefly, NRK-52E cells were collected, washed twice with cold PBS and then resuspended in binding buffer. The cells were subsequently incubated with 10 μl Annexin V-FITC and 5 μl PI for 5 min at room temperature in the dark, followed by analysis by flow cytometry.

Immunohistochemical analysis in NRK-52E cells. The NRK-52E cells were inoculated in a 96-well plate at a cell density of 10^6 cells/well. The NRK-52E cells were fixed with 4% paraformaldehyde and incubated with rabbit anti-Bcl-2 (1:1,000 dilution; A2212; ABclonal) or rabbit anti-Fas (1:500 dilution; A2639; ABclonal) antibodies overnight at 4˚C. Next, cells were washed three times with PBS, incubated with the anti-rabbit secondary antibody (1:100; sc-3753; Santa Cruz Biotechnology, Inc.) for 30 min at 20˚C and then visualized by addition of DAB. The AOD was calculated in five random fields per slide using Image-Pro Plus software (version 5.0) and presented as the mean of three independent measurements.

Western blot assay in NRK-52E cells. The cells were washed three times with PBS, trypsinized, suspended in DMEM and then centrifuged at 3,000 x g for 5 min at 4˚C to remove the supernatant. The total proteins extracted were examined via western blot analysis. Briefly, 40 μg protein from each sample was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Next, the membrane was blocked with 5% non-fat milk in TBST buffer and incubated at 4˚C overnight with the following primary polyclonal antibodies: Anti-Bcl-2, anti-Fas/FasL and anti-GAPDH (dilution, all 1:500). Following three washed with TBST for ~15 min, the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase (dilution, 1:2,000), followed by further washing with TBST for three times. Specific bands were visualized using an enhanced chemiluminescence detection kit, and the band intensity was detected using the Quantity One software.

Statistical analyses. All data are expressed as the mean ± standard deviation. Statistically significant differences between groups were tested by analysis of variance, and all statistical analyses were processed by SPSS version 11.0 statistical software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was regarded as an indicator of statistically significant differences.

Results

Serum BUN and Cr Levels. The levels of the renal function parameters BUN and serum Cr in rats of the various groups are shown in Fig. 1A and B. Compared with the sham surgery rats, the I/R group demonstrated a significant increase in BUN and Cr levels ($P<0.01$). However, the renal function of rats

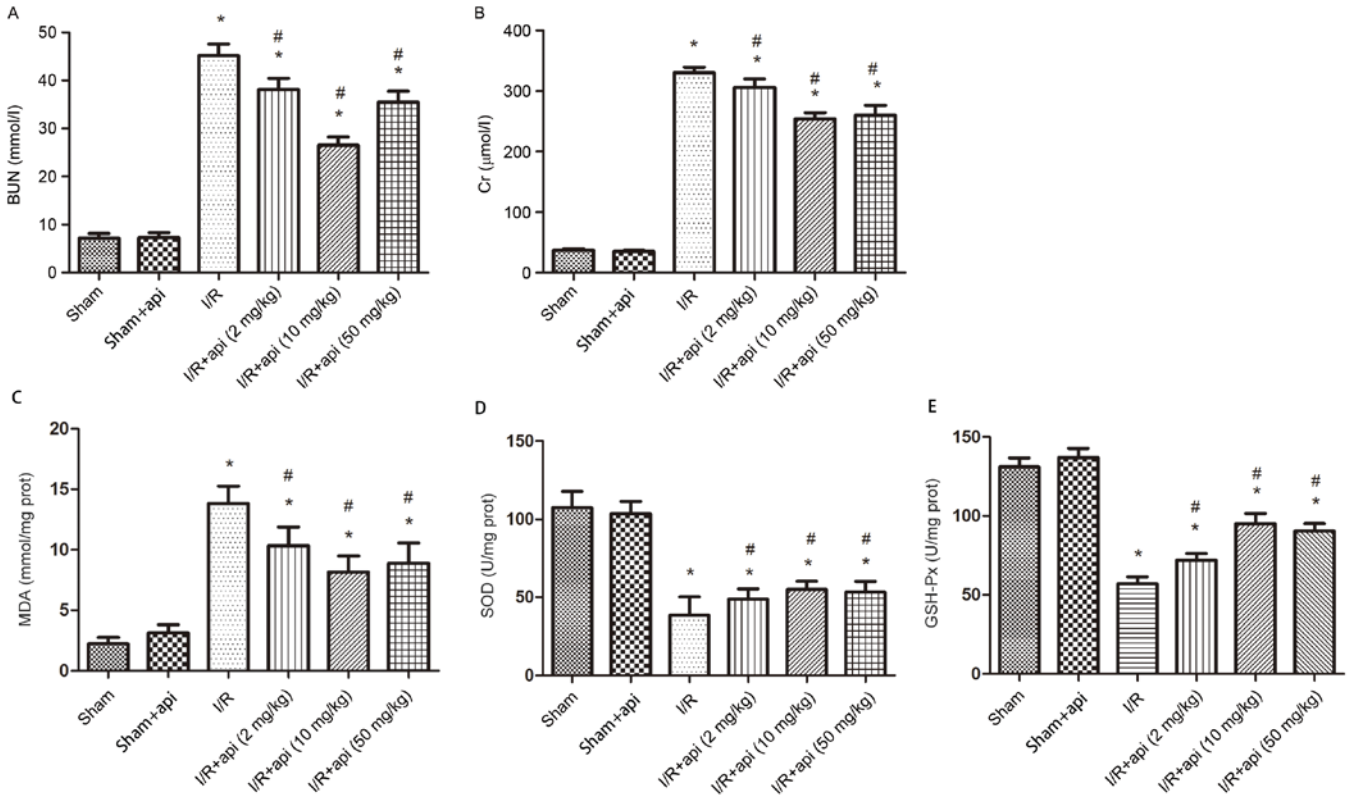

Figure 1. Effect of apigenin on the renal function and anti-oxidative stress of rats. Effect of apigenin on the (A) BUN, (B) Cr, (C) MDA, (D) SOD and (E) GSH-Px levels after 45 min of ischemia. Bars represent the means ± standard deviation (n=6). *P<0.01 vs. sham group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; api, apigenin treatment; BUN, blood urea nitrogen; Cr, creatinine; MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

subjected to I/R was improved by treatment with apigenin as observed by the significantly reduced levels of BUN and Cr in the I/R + apigenin groups compared with I/R alone groups (P<0.05). In addition, the difference in the BUN and Cr levels between the apigenin + sham and the sham-operated group was not statistically significant. Furthermore, the BUN and Cr levels of the I/R + api (2 mg/kg) group was higher than in other apigenin treatment groups (10 and 50 mg/kg), which indicated that the concentration of 10 and 50 mg/kg apigenin were the most effective at reducing I/R injury (Fig. 1).

Measurement of MDA, SOD and GSH-Px. As shown in Fig. 1C-E, the I/R group exhibited a significant increase in MDA (P<0.01), a marker of lipid peroxidation, as well as significant decreases in the SOD and GSH-Px levels compared with the sham group (P<0.01). By contrast, apigenin pretreatment in I/R rats significantly inhibited the decrease in the SOD and GSH-Px levels compared with the I/R only group (P<0.05). Furthermore, no significant difference was identified between the sham and apigenin + sham groups (P>0.05; Fig. 1C-E). Similarly, high doses of apigenin exerted a marked increase in renoprotection against renal I/R injury compared with lower doses of apigenin.

Morphological features of kidneys and immunohistochemical staining. In the I/R group, various morphological abnormalities were identified by H&E staining, including tubular cell necrosis, cytoplasmic vacuolization and tubular lumen obstruction and impairments. However, apigenin pretreatment relieved the severe renal damage caused by I/R injury. Quantitative analysis indicated that 45 min of renal ischemia followed by 24 h of reperfusion resulted in severe acute tubular necrosis, but the Jablonski histological scores in the I/R + apigenin groups were significantly lower than those in the I/R group, which indicated that this renal damage was attenuated by apigenin (P<0.05; Fig. 2A and B).

Immunohistological staining for Bcl-2, Fas and FasL was also conducted in the kidney tissues (Fig. 2A, C and D). The results indicated that Fas/FasL expression significantly increased in the I/R group compared with the sham group (P<0.01), and this tendency was suppressed by apigenin treatment (P<0.05). The immunohistochemical results of Fas gene were almost identical with those for FasL, thus only the immunohistochemical results for FasL are illustrated in Fig. 2. In contrast to the Fas/FasL results, Bcl-2 expression significantly decreased in the I/R group compared with the sham group (P<0.01); however, apigenin pretreatment significantly improved this expression (P<0.05).

Bcl-2, Fas and FasL mRNA expression levels in the kidneys. RT-qPCR was used to investigate the differences in the mRNA expression levels of Bcl-2, Fas and FasL. As shown in Fig. 3A, the expression of Bcl-2 in kidney tissues at the mRNA level demonstrated a significant decrease in the I/R group compared with that in the sham group (P<0.01). However, apigenin evidently improved the mRNA expression of Bcl-2 in the I/R + apigenin groups, since an increased level was observed compared with the I/R group (P<0.05). On the

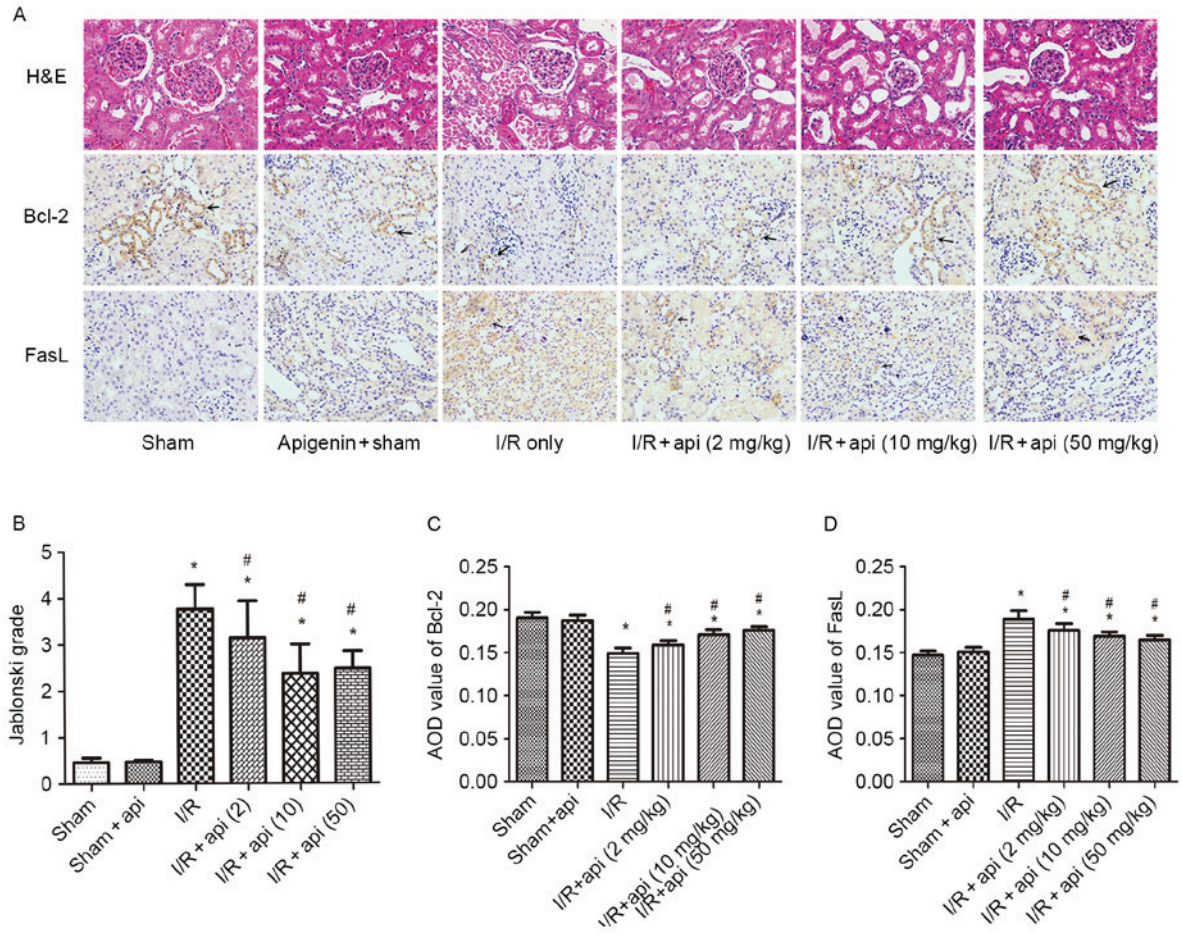

Figure 2. Histological features in rat renal tissues were evaluated by H&E and immunohistochemical staining to examine the expression levels of Bcl-2 and FasL. (A) H&E and immunohistochemical staining images (original magnification, x400). (B) Jablonski scores for the histological appearance of acute tubular necrosis. The AOD of (C) Bcl-2 and (D) Fas are demonstrated. Bars represent the means ± standard deviation (n=6). *P<0.01 vs. sham group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; api, apigenin treatment; Bcl-2, B-cell lymphoma 2; FasL, Fas ligand; AOD, average optical density.

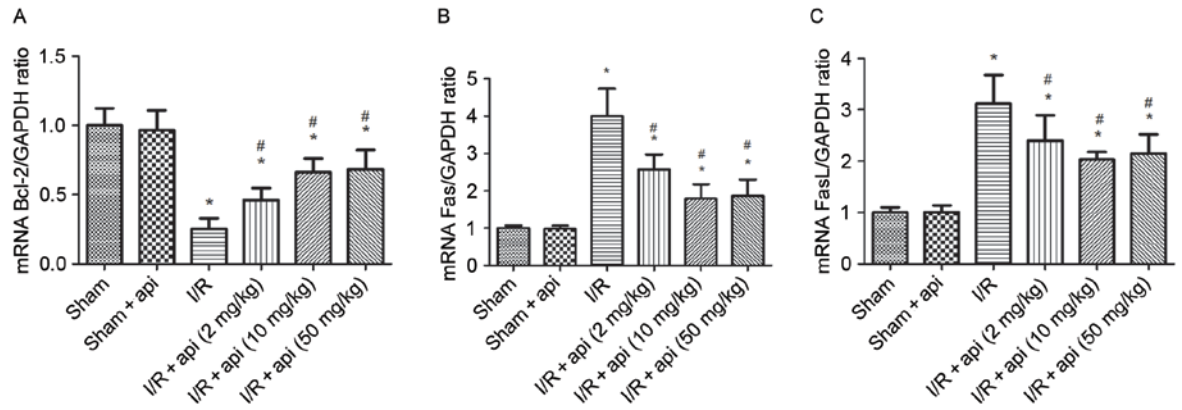

Figure 3. Effect of apigenin on the mRNA expression levels of (A) Bcl-2, (B) Fas and (C) FasL in the rat kidneys after 45 min of ischemia, followed by 24 h of reperfusion. Bars represent the means ± standard deviation (n=6). *P<0.01 vs. sham group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; api, apigenin treatment; Bcl-2, B-cell lymphoma 2; FasL, Fas ligand.

contrary, the mRNA levels of Fas and FasL were significantly higher in I/R group in comparison with the sham group (P<0.01), while apigenin pretreatment inhibited the mRNA expression of Fas and FasL in the I/R + apigenin group compared with the I/R only group (P<0.05; Fig. 3B and C).

Similarly, western blot analysis revealed a significant increase in Fas and FasL protein expression levels in the I/R group compared with the sham group (P<0.01), and these levels were decreased by apigenin pretreatment in the I/R + apigenin groups (P<0.05). Furthermore, as compared with the sham-operated group, the I/R group induced a significant decrease in Bcl-2 protein expression (P<0.01), whereas these effects were antagonized in the I/R + apigenin groups (P<0.05; Fig. 4). The western blot analysis results were consistent with the

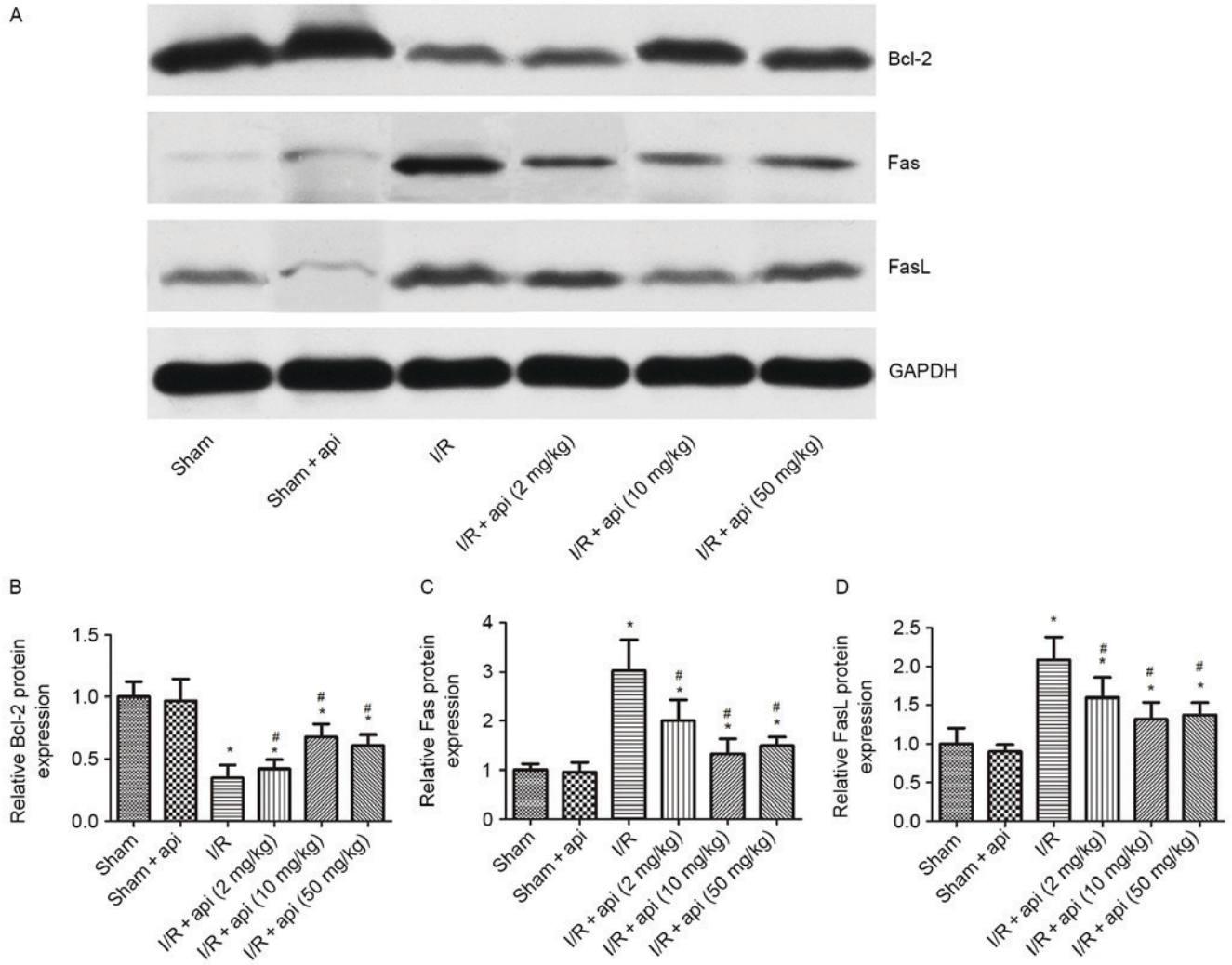

Figure 4. Bcl-2, Fas and FasL protein expression levels in rat renal tissues after 45 min of ischemia, followed by 24 h of reperfusion. (A) Representative western blots showing the effects of apigenin treatment on Bcl-2, Fas and FasL expression levels. Quantified expression levels of (B) Bcl-2, (C) Fas and (D) FasL, relative to the mean value of the sham group. Bars represent the means ± standard deviation (n=6). *P<0.01 vs. sham group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; api, apigenin treatment; Bcl-2, B-cell lymphoma 2; FasL, Fas ligand.

immunohistochemical findings on Bcl-2 and Fas/FasL expression levels. Furthermore, apigenin was indicated to increase Bcl-2 expression and decrease Fas/FasL expression significantly in a dose-dependent manner. The Bcl-2 expression was increased to the highest at a final concentration of 10 and 50 mg/kg, which indicated that apigenin protected kidneys against I/R injury.

Effects of apigenin on NRK-52E cell viability. In order to further investigate the effects of apigenin, *in vitro* experiments were also conducted in an I/R cell model established by anoxia/reoxygenation. The effects of apigenin on the viability of NRK-52E cells induced by I/R were determined quantitatively with a CCK8 assay. I/R in cells significantly decreased the cell survival rate as compared with the normal group. However, apigenin pretreatment prior to I/R significantly increased the viability of NRK-52E cells (Fig. 5). In addition, the effect of apigenin was dose dependent, since the viability of NRK-52E cells was the highest at the final concentration of 1,000 nM apigenin, although the difference among the 1,000 and 100 nM doses was not statistically significant (Fig. 5). Thus, the concentration of 1 μM apigenin

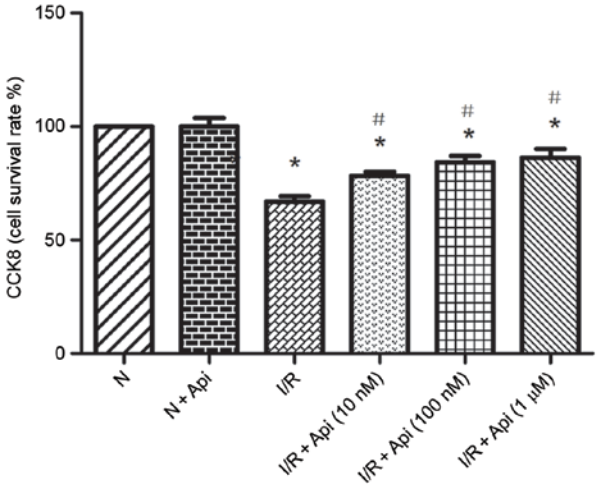

Figure 5. Effects of apigenin on the viability of NRK-52E cells subjected to I/R injury, examined by CCK-8 assay. NRK-52E cells without any treatment served as the N group. At 24 h before I/R, NRK-52E cells were treated with various concentrations of apigenin (10, 100 and 1,000 nM). Bars represent the means ± standard deviation (n=6). *P<0.01 vs. normal group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; N, normal; api, apigenin treatment; CCK-8, cell counting kit-8.

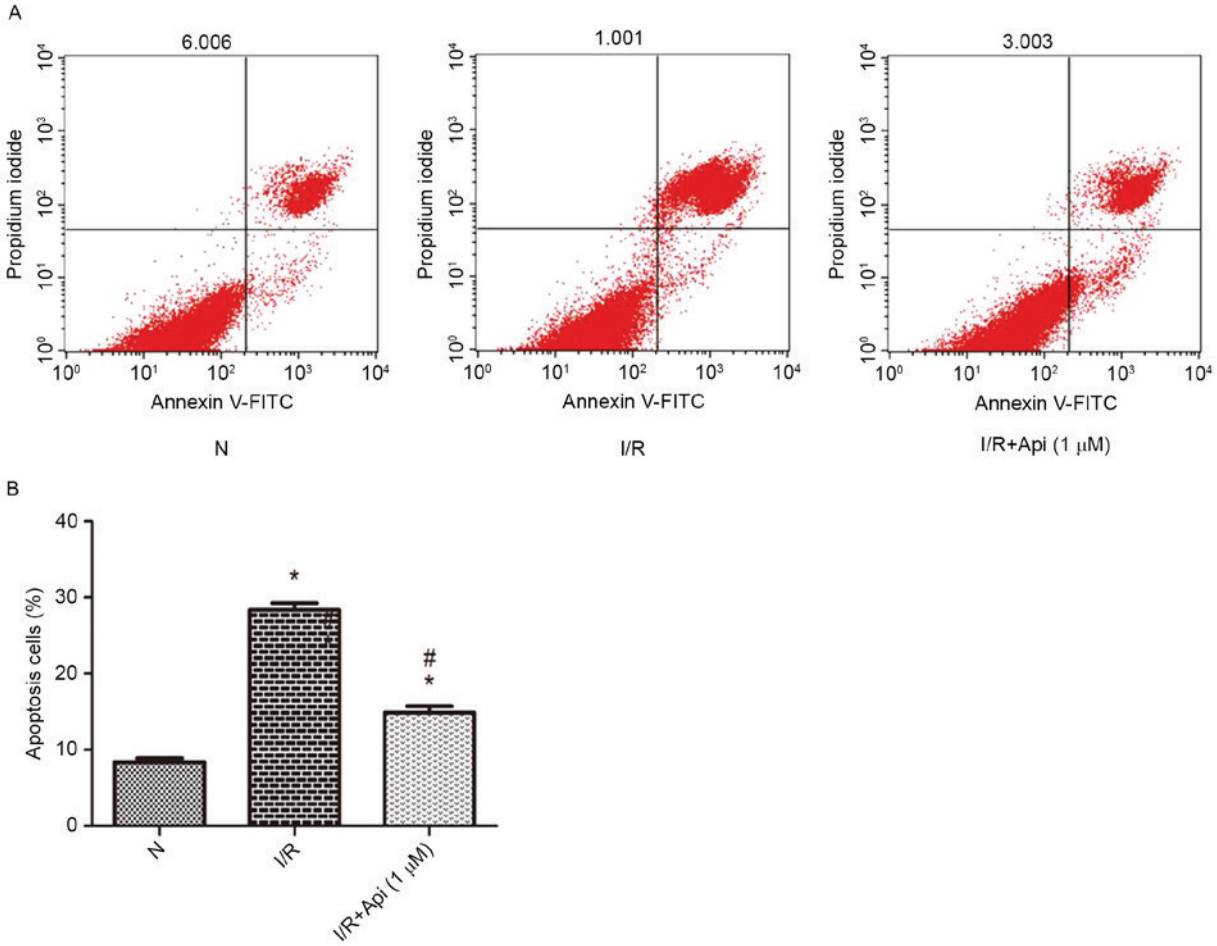

Figure 6. Effects of apigenin on the apoptosis of the NRK-52E cells. (A) Flow cytometry assays demonstrated the apoptosis of the NRK-52E cells treated with apigenin (1,000 nM). (B) Quantification of apoptosis rate in the NRK-52E cells. Bars represent the means ± standard deviation (n=6). *P<0.01 vs. normal group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; N, normal; api, apigenin treatment.

was used in subsequent experiments. In addition, there was no evident difference in cell viability between the normal and normal + apigenin groups, therefore, only the normal group was used in subsequent experiments.

Effects of apigenin on apoptosis in NRK-52E cells. Flow cytometry was conducted to determine whether apigenin was able to inhibit the apoptosis of NRK-52E cells. Compared with the normal group, the I/R group demonstrated significantly increased cell apoptosis. However, the cell apoptosis induced by I/R was reduced upon pretreatment with apigenin (Fig. 6).

Bcl-2, Fas and FasL protein expression levels in NRK-52E cells. To determine the effect of Bcl-2 and Fas/FasL in NRK-52E cells, immunohistochemical analysis was performed. As shown in Fig. 7, the I/R group displayed low protein expression of Bcl-2 and strong protein expression of Fas compared with the normal group. By contrast, pretreatment with apigenin at a concentration of 1,000 nM significantly increased the Bcl-2 protein levels and decreased the Fas protein levels in the I/R + apigenin (1,000 nM) group when compared with the I/R group (Fig. 7). Furthermore, western blot analysis was conducted to analyze the expression levels of Bcl-2 and Fas/FasL. The results indicated that the protein expression levels of Fas and FasL were significantly increased, while the Bcl-2 level was significantly decreased, as compared with the normal group. In addition, pretreatment with apigenin at a concentration of 1,000 nM attenuated the increase in Fas/FasL and the decrease in Bcl-2 expression levels (Fig. 8).

Discussion

Renal I/R injury associated with kidney transplantation and nephrectomy are unresolved problems in clinical practice (25). Acute renal failure can be induced by the renal I/R injury, and it has been suggested that cell apoptosis and necrosis are the main features of this disease (26). Cell apoptosis serves an important role in the process of cell development and the internal environment homeostasis (27). Dysfunction of the apoptosis regulatory mechanism results in a variety of clinical diseases, including neurodegenerative diseases, autoimmune diseases, hematopoietic dysfunction, infertility and cancer (28-32). A large number of animal experiments have demonstrated that renal tubular cell apoptosis can be observed subsequent to kidney damage caused by ureteral obstructive diseases, renal artery stenosis, biological toxin exposure and trauma (33-36). Furthermore, renal I/R injury can induce renal tubular epithelial cell apoptosis and necrosis (37). These two modes exist in kidney pathology as interdependent phenomena resulting from the activation of shared pathways and signals (38).

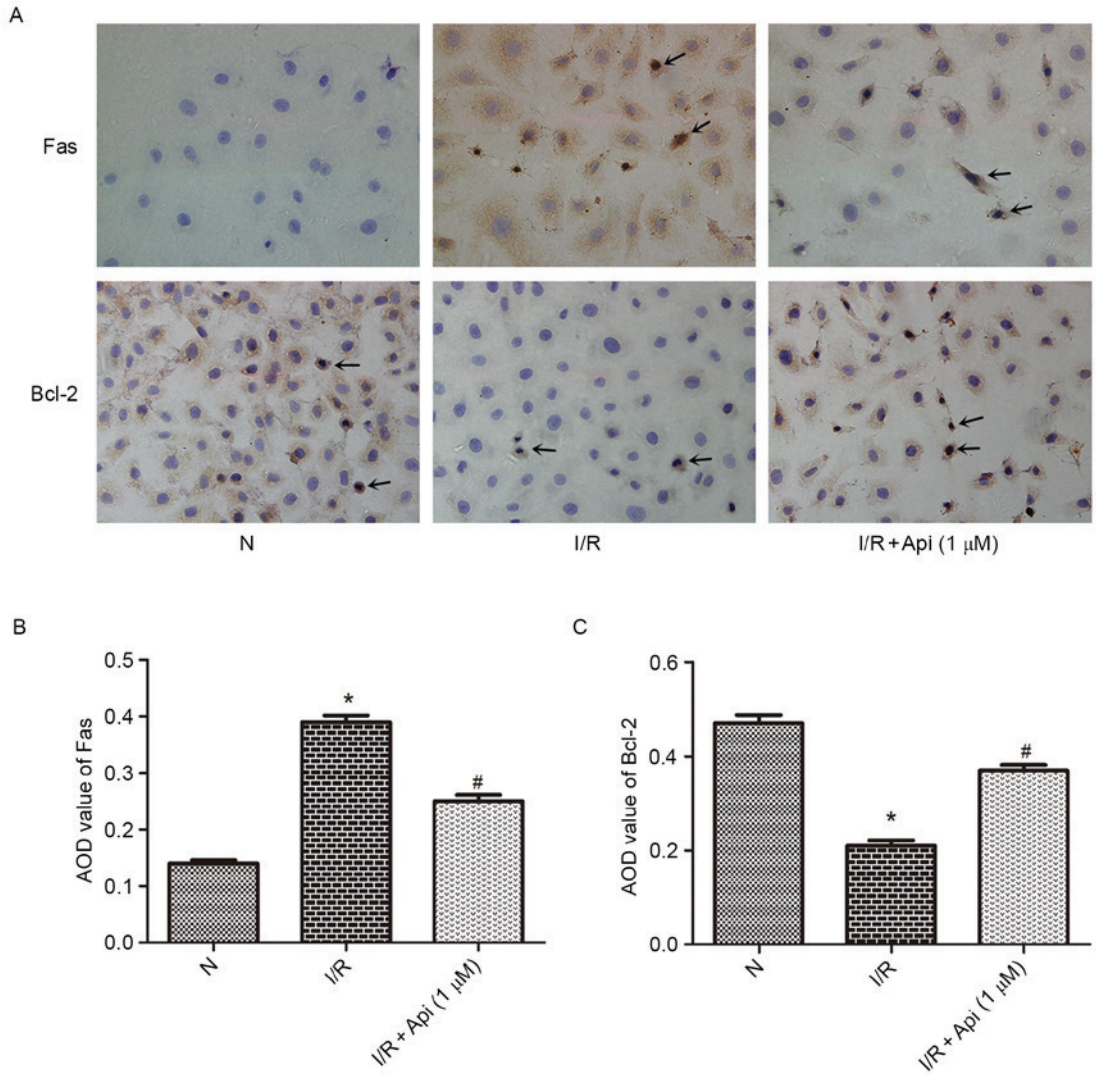

Figure 7. Immunohistochemical analysis examining the expression levels of Fas and Bcl-2 in NRK-52E cells. (A) Fas and Bcl-2 expression levels analyzed by immunohistochemical staining (original magnification, x400). The AOD values of (B) Fas and (C) Bcl-2 were measured. Bars represent the means ± standard deviation (n=6). *P<0.01 vs. normal group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; N, normal; api, apigenin treatment; AOD, average optical density; Bcl-2, B-cell lymphoma 2.

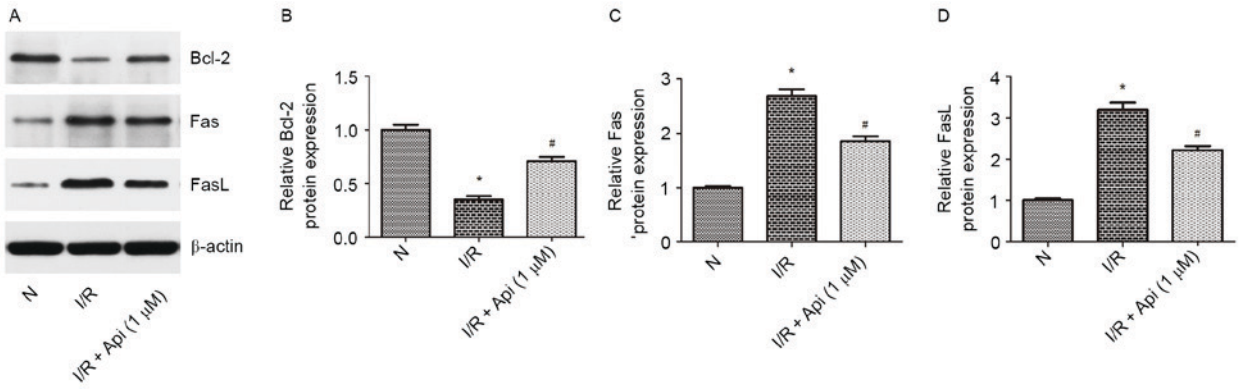

Figure 8. Effects of apigenin on the expression levels of Bcl-2, Fas and FasL in NRK-52E cells exposed to I/R, examined by western blot analysis. (A) Representative western blots demonstrated the effects of apigenin treatment. Quantified band densities of (B) Bcl-2, (C) Fas and (D) FasL, relative to the mean value of the N group. Bars represent the means ± standard deviation (n=6). *P<0.01 vs. normal group; #P<0.05 vs. I/R group. I/R, ischemia-reperfusion; N, normal; api, apigenin treatment; Bcl-2, B-cell lymphoma 2; FasL, Fas ligand.

The present study demonstrated that apigenin, a common edible plant flavonoid and a well-characterized antioxidant (39), had a renoprotective effect by inhibiting apoptosis in renal I/R injury. However, the mechanisms underlying cell apoptosis are

complicated. To clarify the possible mechanisms mediating the anti-apoptotic effect of apigenin, the expression levels of Bcl-2 and Fas/FasL, as well as the levels of MDA, SOD and GSH-Px, were investigated in the current study. The results revealed that the expression of Bcl-2 was decreased by I/R injury and improved by apigenin treatment, while the opposite expression alterations were observed for Fas/FasL. Furthermore, apigenin exhibited protective effects by reducing the oxidative stress through reduced production of free radical derivatives and increased production of antioxidants, as evidenced by the decreased MDA level and the increased levels of SOD and GSH-Px. These results suggest that apigenin inhibits renal cell apoptosis and protects against the oxidation of renal cellular membrane damage by enhancing the activation of the Bcl-2 pathway and blocking the activation of the Fas/FasL pathway in renal I/R injury. Furthermore, the current data indicated that the 10 and 50 mg/kg treatment subgroups exhibited a marked renoprotective effect as compared with the 2 mg/kg treatment.

In order to examine the toxicity of apigenin, the renal function and protein levels of Bcl-2, Fas and FasL in the sham surgery and apigenin + sham groups were first compared. No significant differences were observed between these two groups in terms of the BUN, serum Cr, Bcl-2, Fas and FasL protein levels ($P>0.1$), indicating that apigenin was non-toxic for normal renal tissues.

The Bcl-2 family is one of the primary regulatory factors of cell apoptosis (40), and has received the most attention among a variety of relevant proteins functioning in apoptosis regulation (41). Bcl-2 is one of the most important anti-apoptotic genes, and is expressed at low levels in renal tissues following I/R (42). When I/R injury was induced in the present study, the amount of apoptotic cells in distal convoluted tubules increased when compared with the sham surgery group. However, compared with the I/R group, Bcl-2 was highly expressed at the distal convoluted tubules of the I/R + apigenin groups. High expression of Bcl-2 serves an important role in the anti-apoptosis mechanism of renal tissues treated with I/R. Thus, the present study suggested that apigenin regulated the expression of the apoptosis-inhibitory Bcl-2 gene in I/R injury. The anti-apoptosis mechanism of Bcl-2 may be associated with several factors: i) Bcl-2 functions as an anti-apoptotic protein causing endoplasmic reticulum Ca^{2+} depletion and helping to keep the luminal Ca^{2+} concentration at physiological levels, decreasing the cellular Ca^{2+} concentration by inhibiting Ca^{2+} release (43); ii) cell apoptosis gene signaling and apoptosis-associated gene products are blocked by Bcl-2; iii) oxidative stress triggers multiple signaling pathways, including the pathways involving the Bcl-2 proteins. A previous study suggested that the upregulated expression of Bcl-2 can lead to the decrease of oxidative stress (44). Bcl-2 gene is a terminal part of apoptotic regulation, and high expression of Bcl-2 protein may reduce the formation of lipid peroxide and oxygen free radicals. Thus, Bcl-2 is an important survival factor, which is sensitive to oxidative stress and an increase in Bcl-2 suppress cell apoptosis.

Fas, a transmembrane protein that belongs to the TNF superfamily, is one of the death receptors in the cell membrane (45). Death receptors and the apoptotic cascade are activated upon engagement with the corresponding ligand, which is FasL in the case of Fas (46). Fas/FasL are considered as cell apoptosis genes, and the combination of Fas and its ligand result in Fas-associated cell apoptosis (47). In addition, FasL, expressed in renal tubular epithelial cells of normal rat kidney, may accelerate the apoptosis of lymphocytes (48). The apoptosis mechanism of Fas/FasL may be associated with the overexpression of the immediate early gene, immunological dysfunction and the effect of certain inflammatory cytokines. In the present experimental study, the expression of the Fas protein was significantly increased following I/R injury, in comparison with the sham surgery group. Similar results were observed for the expression of FasL protein. Thus, Fas/FasL induced cell apoptosis in the kidney following the induction of I/R injury. Furthermore, the data demonstrated that there was a significant difference between the I/R and I/R + apigenin groups. It was observed that apigenin was able to block the interaction between Fas and FasL, strongly inhibiting renal injury after I/R. The Fas system may be involved in the triggering of renal tissue cell apoptosis by oxidative stress. Thus, the present study also validated the effect of oxidative stress on membrane Fas/FasL expression in renal tubular epithelial cells. Nevertheless, the mechanism of Fas/FasL expression induced by oxidative stress remains unclear. A possible mechanism of oxidative stress-induced Fas/FasL expression is the tyrosine phosphorylation of signaling molecules, including p38 mitogen-activated protein kinase and Jun-N-terminal kinase, which have been implicated in Fas/FasL expression (49).

In conclusion, the results of the present study revealed that apigenin increased the expression of Bcl-2 and reduced Fas/FasL expression in renal I/R injury, providing marked protection against this injury in rats. The present study also suggested that the upregulation of Bcl-2 and downregulation of Fas/FasL protein expression levels are involved in anti-apoptosis and antioxidation, and may be one of the mechanisms underlying the protective effect of apigenin on renal I/R injury.

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