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# Probucol Protects Against Contrast-Induced Acute Kidney Injury via the Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2)/JNK-Caspase 3 Pathway in Diabetic Rats

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Statistical Analysis C  
Data Interpretation D  
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**Background:** Contrast-induced acute kidney injury is an important clinical problem, yet its pathogenic mechanisms are incompletely understood. In this study we explored the potential beneficial effects of probucol as treatment of contrast-induced acute kidney injury in diabetic rats.

**Material/Methods:** Rats were divided into 3 groups: i) diabetic control, ii) diabetic with contrast, and iii) probucol treatment groups. Probucol was administered by gavage and the contrast diatrizoate (60%) was injected via femoral vein. After 24 h, the rats were sacrificed and samples were taken to measure biochemical indicators. Pathological damage of renal tubules was evaluated by HE staining. Expression of Bcl-2, Bax, p-ERKs, and p-JNK proteins in the kidneys was examined by Western blotting, whereas expression level of caspase-3 in kidneys was detected by immunohistochemistry.

**Results:** Compared to the probucol treatment group, the diabetes with contrast group showed higher serum creatinine and lower creatinine clearance. The pathological changes of kidneys in the probucol treatment group were improved compared with the contrast group. Moreover, Western blot analyses revealed that use of contrast agent led to lower p-ERK1/2, higher p-JNK, lower Bcl-2, and higher Bax levels, which were reversed by probucol. Finally, immunohistochemical findings revealed higher caspase-3 after contrast use, which was partially reversed by probucol.

**Conclusions:** Probucol exerts protective effects on contrast-induced acute kidney injury in diabetic rats by inhibition of renal cell apoptosis. This is achieved by reducing mitochondrial caspase-3 expression through increasing and decreasing the expression of the upstream mediators p-ERK1/2 and p-JNK, respectively.

**MeSH Keywords:** **Acute Kidney Injury • Apoptosis • Diabetes Mellitus • Probucol**

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## Background

The definition of contrast agent acute kidney injury is impairment in renal function (an increase in serum creatinine by more than 25%) occurring within 3 days following the intravascular administration of contrast media and the absence of alternative etiology [1]. Contrast-induced acute kidney injury is one of the 3 major causes of hospital-acquired acute renal failure, particularly in those receiving percutaneous coronary intervention [2,3]. Diabetic nephropathy is the most common risk factor for contrast-induced acute kidney injury [4,5]. Cellular studies found that apoptosis is significantly aggravated in subjects with hyperglycemia (30 mmol/l) after administration of hypertonic or hypotonic contrast media [6]. This suggests a potential synergistic effect between them. Probucol is a lipid-lowering agent with pleiotropic effects, including antioxidative and anti-inflammatory properties [7] and even anti-apoptosis effects [8–10]. It can also improve endothelial function and inhibit vascular intimal hyperplasia. Previously, our group reported that probucol can prevent the occurrence of contrast-induced acute kidney injury after coronary intervention in patients with chronic renal failure, reducing its incidence from 15% to 8% [11].

Recently, studies have shown that apoptosis [12,13] and the balance between p-ERK and p-JNK are linked to the pathogenesis of contrast agent acute kidney injury [14], and apoptosis is inhibited and promoted by activation of the ERK and JNK signaling pathways, respectively [15]. However, whether the protective effect of probucol on tubular epithelial cell mediated by apoptosis is associated with the activation of the ERK and JNK in the pathogenesis of contrast agent acute kidney injury in diabetic rats is still uncertain. This study investigated the involvement of apoptosis and ERKs and JNK in the process of contrast-induced acute kidney injury in diabetic rats, as well as the molecular mechanism underlying renoprotective effect afforded by probucol.

## Material and Methods

### Animals

Eighteen healthy male Sprague-Dawley rats (SPF grade) weighing  $250 \pm 20$  g were purchased from the Academy of Military Medical Sciences. All rats were housed in a 12-h light/12-h dark cycle at  $22\text{--}25^\circ\text{C}$  with free access to standard diet and tap water. The design and experimental procedures of the study were approved by the Medical Ethics Committee of Tianjin Medical University (SYXK (Jin)-2009-0001) and complied fully with the protocol outlined in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Experimental design and drugs

All rats adapted to the environment for 1 week prior to experimentation. Rats received intraperitoneal injection of 1% STZ (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) dissolved in citrate buffer (PH 4.5) after 12 h fasting at a single dose of 60 mg/kg to establish a diabetic model. Afterwards, they had free access to standard diet and water. Blood samples were taken from the tail of the rats to measure the blood glucose 1 week after injection of STZ. Successful induction of diabetes was defined as blood glucose  $\geq 16.7$  mmol/L 1 week after STZ injection. Subsequently, the diabetes mellitus rats were randomly divided into 3 groups and were kept for another 7 weeks: a diabetes control group (n=6), a diabetes with contrast group (n=6) and a probucol treatment group (n=6). In the ninth week, 5% probucol (Shandong Qilu Pharmaceutical Co., Shandong, China) was made by dissolving in 0.5% carboxymethylcellulose and was given by oral gavage to the probucol treatment group rats at a dose of 500 mg/kg for 14 consecutive days. In the control group and contrast only group, the rats received an equal volume of 0.5% carboxymethylcellulose by gavage once daily on the same days. At the end of 10 weeks, 60% diatrizoate was used as the contrast agent (Shanghai Xudong Haipu Pharmaceutical Co., Shanghai, China) to establish the diabetic rat model of acute kidney injury. It was administered intravenously at a dose of 10 mL/kg into the femoral vein to rats in the diabetes with contrast group and the probucol treatment group with a single intraperitoneal injection of pentobarbital (30 mg/kg) once daily for 2 consecutive days. The diabetes control group was injected with the same amount of normal saline in the same way as the control group. Finally, rats were sacrificed and the kidneys were obtained for use in subsequent experiments. In the process of making diabetes models, blood glucose was measured again at weeks 8 and 10, 1 rat died accidentally on the third day after STZ injection and 1 new rat was subsequently added.

Drug administration and collection of blood and urine samples were performed between 8:00 and 9:00 a.m. to minimize circadian variations. All drugs are prepared when needed. All samples were kept at  $-80^\circ\text{C}$  until required for analysis. To collect 24-h urine, rats were kept in individual metabolic cages. Blood samples were taken from the caudal vein or inferior vena cava, and both kidneys were excised at the end of the study. One kidney was fixed in 10% neutral formalin and dehydrated, embedded in paraffin, sectioned, baked, and prepared for use with relevant pathological staining, while the other kidney was frozen with liquid nitrogen and then transferred to a freezer at  $-80^\circ\text{C}$  for examination.

### Blood glucose and renal function assessment

Blood glucose levels were measured by glucose meter and glucose test strip (Johnson & Johnson, New Jersey, USA) before and at 1, 8, and 10 weeks after STZ injection.

The serum creatinine (Cr) and urinary creatinine (Ucr) levels were measured using an automatic biochemical analyzer based on the Creatinine Kit (Biosino Biotechnology & Science Co., Beijing China.) instructions. Creatinine clearance (CrCl) was calculated by  $U \times V / P$  [where U=urine creatinine (mg/dl), V=urine volume (ml/min/100 g), and P=serum creatinine (mg/dl)], and was expressed as ml/min/100 g body weight.

### Pathological damage of renal tubules was evaluated by HE staining

HE staining was performed. The renal tissue in 10% neutral formalin were dehydrated, embedded in paraffin, sectioned (4–5  $\mu\text{m}$ ), dewaxed in xylene, and placed in a 60°C oven for 60 min. Tissue sections were dehydrated by different alcohol concentration gradients, then stained with hematoxylin, put into 1% hydrochloric acid ethanol, infiltrated with eosin, and dehydrated again by alcohol gradients of different concentrations. Finally, tissue sections were made transparent by xylene and sealed by glass slide covers.

Microscopy was used to determine if the cytoplasm is pink and the nucleus is blue-violet. In severe lesions, the Paller method was used to assess the injury degree of renal tubules [16], in which 10 diseased renal tubules were randomly selected at each high power (HP, 400 $\times$ )-field and scored according to 100 tubules. A markedly expansive renal tubule and flat cell were scored 1 point, damage or shedding of renal tubular brush border was recorded as 1 or 2 points, respectively, tube cast was scored 2 points, necrotic and shedding cells not yet forming a tube type in the lumen were scored as 1 point. The average renal tubular injury score under each field of view was calculated as the renal tubular injury score of the group.

### Western blotting analysis expression levels of GAPDH, Bcl-2, Bax, and upstream p-ERK1/2 and p-JNK protein

The frozen kidney tissue mass was taken from the freezer at  $-80^{\circ}\text{C}$ , weighed, and fully ground using liquid nitrogen. Lysis buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to extract kidney tissue proteins of rat. Concentration of protein was tested using Coomassie Brilliant Blue. We added a 20- $\mu\text{g}$  protein sample to the SDS-PAGE gel and electrophoresed and transferred it to the PVDF membrane (GE Healthcare Life Sciences, China). The membranes were blocked with 5% milk (dissolved in 1% TBS-T solution) for 2 h, and then incubated overnight at 4°C with the mouse anti-GAPDH (1: 2500; Abcam Inc, Cambridge, UK), rabbit anti-bcl-2 (1: 1000; Abcam, Inc, Cambridge, UK), rabbit anti-Bax (1: 2000; Abcam Inc, Cambridge, UK), rabbit anti-p-JNK (1: 4000; Abcam Inc, Cambridge, UK), and rabbit anti-p-ERK1/2 (1: 4000; Abcam, Inc., Cambridge, UK) antibodies. After incubation and washing, membranes were incubated with corresponding secondary antibodies, anti-mouse/

rabbit IgG (1: 5000; Cell Signaling Technology, Inc.) that bind to horseradish peroxidase (HRP). According to the chemiluminescence image development, Image Lab software (Bio-Rad, Shanghai China) was used to analyze the optical density values of the strip on the film, and to compare the difference of the relative optical density among the various groups after correction.

### Expression of caspase-3 by immunohistochemical detection

The renal tissue was dehydrated in 10% neutral formalin, embedded in paraffin, sectioned (4–5  $\mu\text{m}$ ), hydrated, routinely dewaxed in xylene, and repaired by antigen. Then, in the dark, slices were incubated with 3%  $\text{H}_2\text{O}_2$  deionized water for 10 min to eliminate endogenous peroxidase activity. The caspase-3 rabbit anti-rat polyclonal antibody (1: 100; Abcam, Inc., Cambridge, UK) and the corresponding biotin-labeled goat anti-rabbit IgG (Zhong Shan Jinqiao Biotechnology Co., Beijing, China) were then added dropwise. Phosphate buffer instead of primary antibody was used as a negative control. DAB was stained at room temperature. The positive expression was brownish yellow and observed under a light microscope. Ten complete and non-overlapping fields were randomly measured on each slice. After correcting the optical density, the cumulative optical density (IOD) of the positive reaction was measured in each visual field using Image Pro Plus (Media Cybernetics, USA), and the cumulative optical density represented the content density.

### Statistical analysis

All analyses were performed with SPSS software version 13.0 (SPSS, Inc., Chicago, IL). Data are expressed as means  $\pm$ SD values. Statistical significance ( $P < 0.05$ ) of differences among groups was estimated by an ANOVA followed by least-significant difference (LSD) test or *t* test.

## Results

### Level of blood glucose, body weight, and urine volume in each group

As shown in Table 1, the blood glucose level was much greater than 16.7 mmol/L 1 week after STZ injection, indicating the successful establishment of the diabetes model. In weeks 8 and 10, their blood glucose levels continued to be high, and there was no significant difference between the 3 groups, indicating that probucol had no effect on blood glucose levels in diabetic rats. Moreover, there was no significant difference in body weight or urine volume between rats in each group.

**Table 1.** Comparison of blood glucose, body weight, and urine volume in each group ( $\bar{x}\pm s$ ).

	Diabetes control group (n=6)	Diabetes with contrast group (n=6)	Probucol treatment group (n=6)
BG (mmol/L)			
Before injection	3.81±0.27	3.84±0.36	3.83±0.39
7 days	21.44±2.88	22.17±2.94	22.23±1.93
8 weeks	22.23±3.68	23.02±2.19	22.82±2.56
10 weeks	22.97±2.19	23.48±3.12	23.10±2.48
BW (g)	302.21±45.87	298.54±48.75	289.16±48.54
V (ml/24h)	235.21±30.62	224.48±35.12	233.56±33.89

BG – blood glucose level; BW – body weights; V – urine volume. The blood glucose level was much greater than 16.7 mmol/L at 1, 8, and 10 weeks after STZ injection and there was no significant difference in blood glucose among groups at 10 weeks. Moreover, there was no significant difference in body weight and urine volume between rats in each group.

**Table 2.** Renal function parameters in the study groups ( $\bar{x}\pm s$ )

	Diabetes control group (n=6)	Diabetes with contrast group (n=6)	Probucol treatment group (n=6)
Cr (μmol/L)	71.52±7.03	103.89±9.01 <sup>#</sup>	88.10±8.78 <sup>*</sup>
CrCl (ml/min)	2.60±0.54	1.49±0.33 <sup>#</sup>	2.14±0.49 <sup>*</sup>

Cr – serum creatinine; CrCl – creatinine clearance rate. <sup>#</sup> P<0.05, vs. diabetes control group; <sup>\*</sup> P<0.05, vs. diabetes with contrast group.

### Renal function parameters

The different parameters are shown in Table 2. An increase in the serum creatinine level from 71.52±7.03 to 103.89±9.01 μmol/L and a decrease in the creatinine clearance from 2.60±0.54 to 1.49±0.33 ml/min were observed following injection of hypertonic contrast agent in diabetic rats (P<0.05). These changes were partially prevented by probucol, with creatinine recovering to 88.10±8.78 μmol/L and creatinine clearance to 2.14±0.49 ml/min.

### Evaluation of renal tubular damage in each group by HE staining.

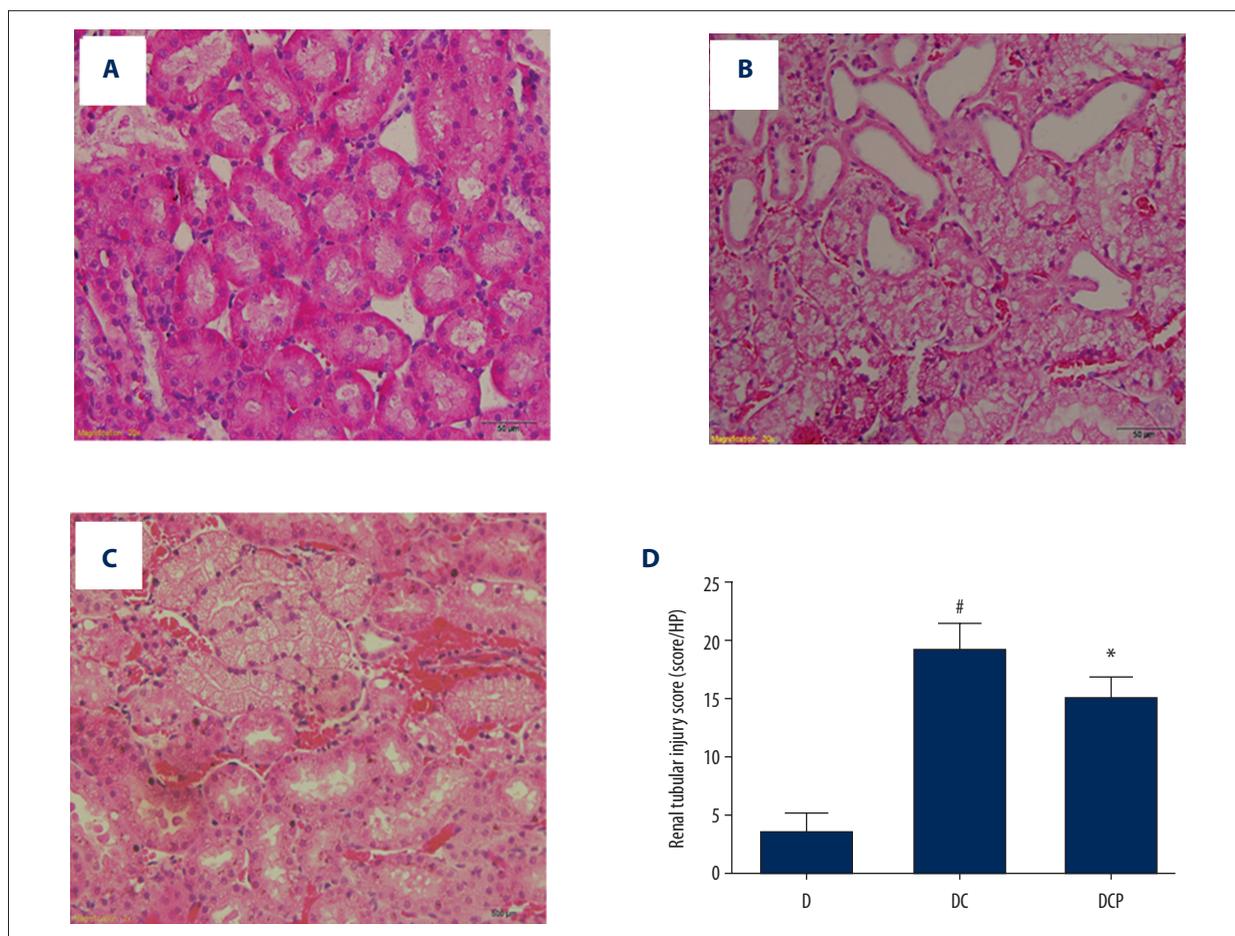
The renal tubules epithelial cells pathological features of DC rats (Figure 1B) were significantly different from those of control group D (Figure 1A), which means the epithelial cells of renal tubules showed extensive vacuole-like changes, and fragmented or necrotic cells that were exfoliated into the lumen of renal tubules, and some of the renal tubule lumens were dilated and degenerated (p<0.05; summarized in Figure 1D). Compared with the DC group, the pathological changes of kidney in the DCP group (Figure 1C) were improved, including vacuolar degeneration of renal tubular epithelial cells and dilatation of lumen (p<0.05). The average renal tubular injury score under each field of view is shown in Figure 1D, which shows that the partial improvement of renal tubular injury was due to probucol (p<0.05).

### Renal expression of ERK1/2, JNK, Bcl-2, Bax, and caspase-3

Next, we tested the hypothesis that caspase-3, an apoptosis-related protein, is a critical mediator of apoptosis in contrast-induced acute kidney injury and this may involve the ERK1/2-JNK-bcl-2 and Bax pathways. Firstly, Western blot analyses revealed that the use of the contrast agent, diatrizoate, led to lower p-ERK1/2 (Figure 2A) and higher p-JNK (Figure 2B) levels, as summarized in Figure 2C. Moreover, lower Bcl-2 (Figure 3A) and higher Bax (Figure 3B) levels were found in the contrast group (p<0.05; summarized in Figure 3C). These changes were prevented by probucol (p<0.05). Finally, immunohistochemical findings for caspase-3 for the diabetic control, diabetic contrast, and probucol treatment groups are shown in Figure 4A–4C, respectively. The integrated optical density values are shown in Figure 4D, which shows that the increase in caspase-3 induced by the contrast (p<0.05) was partially prevented by probucol (p<0.05).

### Discussion

Probucol is a commonly used lipid-regulating drug for the prevention of cardiovascular diseases. Previous studies have focused on the protection of myocardium and vascular endothelial cells due to the lipid-regulating effect of probucol. It has antioxidant properties [17–19] and has been shown to delay the development of diabetic nephropathy [20,21]. Studies have shown

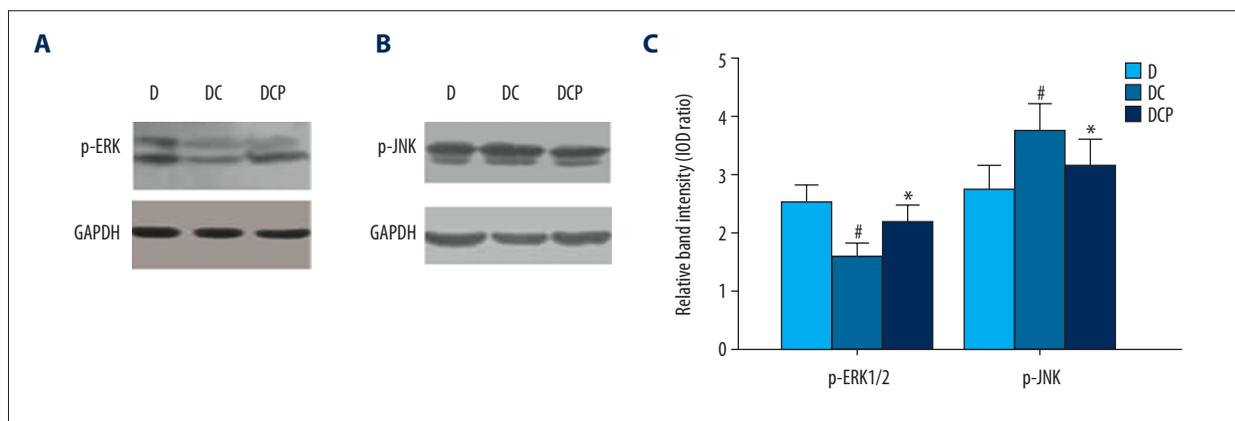


**Figure 1.** (A–D) HE staining evaluated renal tubular damage in each group. The average renal tubular injury score under each field of view was calculated as the renal tubular injury score of the group. #  $P < 0.05$ , vs. diabetes control group; \*  $P < 0.05$ , vs. diabetes with contrast group.

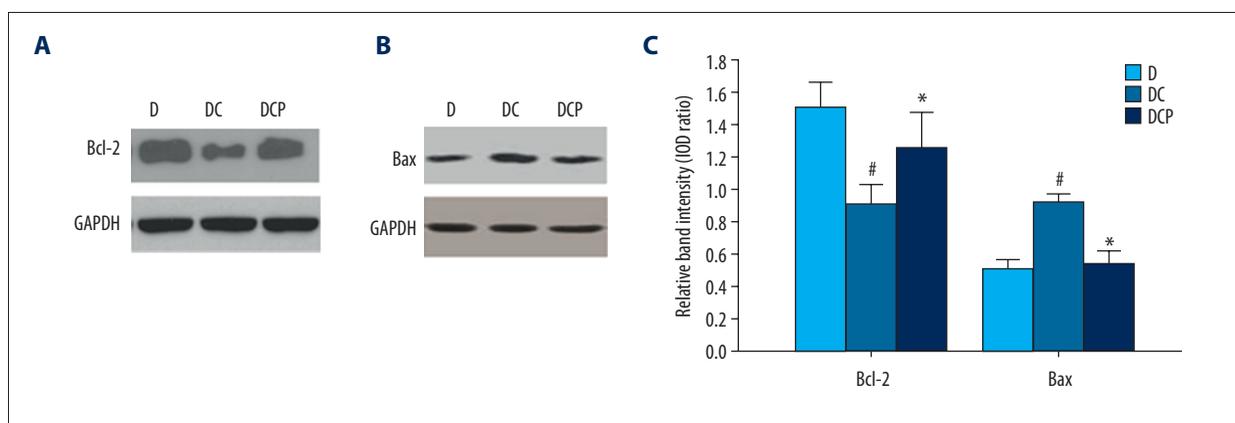
that oxygen free radicals can promote cell apoptosis [22,23]. Probucol accelerates the recovery of renal function and renal pathology by reducing local renal oxidative stress in diabetic rats [20,21,24] and contrast-induced acute kidney injury [25]. Furthermore, research found that probucol can reduce renal oxidative stress in diabetic contrast-induced acute kidney injury and restore the anti-oxidative enzyme activity of glutathione peroxidase in the kidneys [24,26], which can improve the antioxidant defense ability. However, there is limited data on its anti-apoptotic effects on diabetic contrast-induced acute kidney injury. Therefore, we only investigated the anti-apoptotic effect of probucol in diabetic contrast-induced acute kidney injury.

Previous experiments have shown that caspase-3 is a necessary pathway for the cascade of apoptotic proteases [27], and the mitochondrial Bcl-2 family is the most important regulatory factor in the endogenous apoptotic pathway [28]. Iodinated contrast medium can induce apoptosis of renal tubular epithelial cells [29] and caspase-3 is the primary implementer caspase apoptosis [27]. Apoptosis can be initiated through

p53-induced activation of Bax in a caspase-dependent manner [30]. Probucol blocks cyclophosphamide-induced apoptosis by restoring the upregulation of P53 and Bax genes and downregulation in Bcl-2 gene to the normal values [9]. In the present study, we found that the expression of anti-apoptotic protein Bcl-2 was downregulated and the expression of pro-apoptotic protein Bax was upregulated in diabetic rats injected with ionized hypertonic contrast medium. The expression of apoptosis-related protein caspase-3 was significantly upregulated in the contrast group, which suggests that hypertonic contrast medium induces apoptosis in diabetic kidneys via the mitochondrial caspase-3 pathway. The application of probucol significantly reversed the above changes, and resulted in upregulation of anti-apoptotic protein Bcl-2 and downregulation of the pro-apoptotic protein Bax, and caused a significant decrease caspase-3 expression downstream. These results suggest that the ionized hypertonic CM can break the balance between the Bcl-2 and Bax in the Bcl-2 gene family through specific signal transduction cascades, resulting in a decrease in the Bcl-2/Bax ratio when the body is in the basic state of



**Figure 2.** Western blot electrophoresis for p-ERK1/2 (A) and p-JNK (B) in each group. Relative band intensity values are shown in C. # P<0.05, compared to the diabetes control group; \* P<0.05, compared to the diabetes with contrast group.

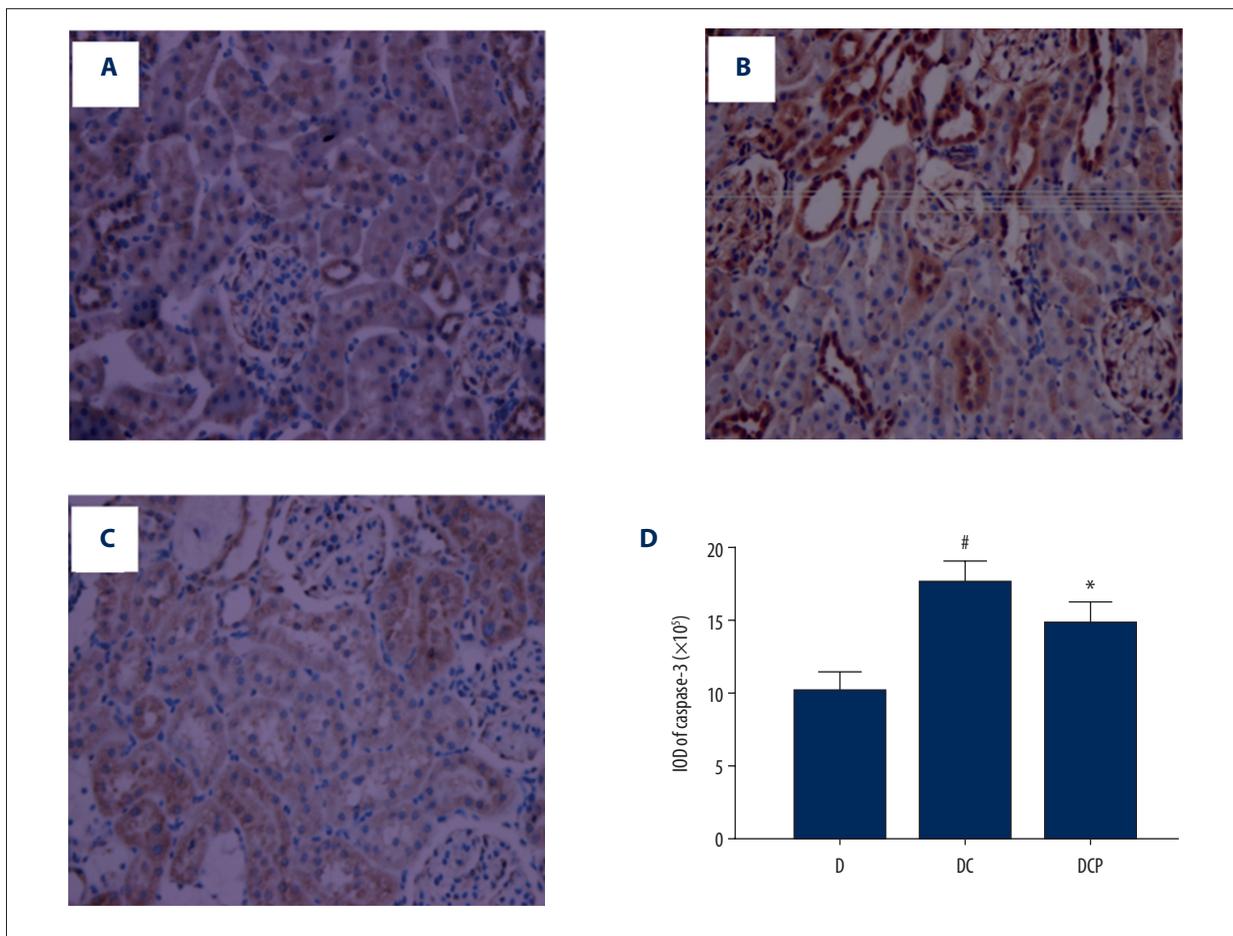


**Figure 3.** Western blot electrophoresis for Bcl-2 (A) and Bax (B). Relative band intensity values are shown in C. # P<0.05, compared to the diabetes control group; \* P<0.05, compared to the diabetes with contrast group.

diabetes, and then the mitochondrial caspase-3 pathway is fully activated to promote apoptosis in the kidneys. However, probenol significantly inhibited activation of the mitochondrial caspase-3 signaling pathway induced by hypertonic contrast medium and alleviated renal cell apoptosis.

The functions of the ERK and JNKs 1 and 2 (ERK1/2) are mediated through phosphorylation, and the dynamic balance between activated ERK and activated JNK may be important in determining whether a cell survives or undergoes apoptosis (i.e., the balance between p-ERK1/2 and p-JNK) [15]. If the balance between p-ERK1/2 and p-JNK is upset in the process of apoptosis, p-ERK is increased and p-JNK is decreased, or vice versa. Apoptosis was inhibited and promoted by activation of the ERK and JNK signaling pathways, respectively [15]. Reactive oxygen species can effectively activate JNK1/2, and the p-JNK1/2 level was correlated with oxidative stress-induced apoptosis [32]. A recent study also confirmed that probenol can reduce cyclophosphamide-induced oxidative apoptosis in rat myocardial tissue, downregulate the expression of p53 and Bax signals, restore Bcl-2 expression, and affect the activity of the

mitochondrial apoptosis pathway [9]. Among these, p53 is regulated by p-ERK [33,34]. Our previous experimental studies showed that phosphorylation of signal molecules ERK1/2 and JNK was involved in renal cell apoptosis induced by iodinated contrast medium in diabetic rats [14]. Our results are consistent with a recent study by Lee et al. [35], which demonstrated that apoptosis is mediated by the phosphorylation of JNK in contrast media-induced nephropathy. Probenol can upregulate P53 and Bax genes and downregulate Bcl-2 in cyclophosphamide-induced oxidative apoptosis [9]. However, no research has been reported on the underlying molecular protection of probenol on apoptosis via activating ERK and inhibiting of JNK signaling proteins, which are located upstream of the p53-induced Bax and Bcl-2 proteins involved in apoptosis [9,33,34]. The present study demonstrates that the level of renal tubular injury score is positively correlated with the inhibition of p-ERK1/2 and the promotion of p-JNK. We also found a significant reduction of p-JNK, increased p-ERK1/2, downregulated Bax, and upregulated Bcl-2 protein expression, reduced serum creatinine level, and amelioration of renal tubular injury pathology in diabetes contrast-induced acute kidney injury rats after treatment with probenol. Our results suggest that

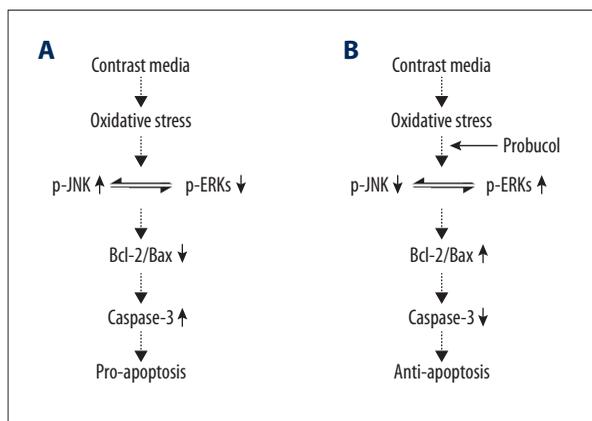


**Figure 4.** Immunohistochemistry findings of caspase-3 for each group (magnification at 400x) (A-C). Integrated optical density values are shown in D. # P<0.05, compared to the diabetes control group; \* P<0.05, compared to the diabetes with contrast group.

probenol is a lipid-regulating drug with anti-apoptosis properties that protect against contrast agent-induced damage in tubular epithelial cells. The mechanism by which probenol protects against apoptosis injury may via the ERK1/2/JNK-caspase 3 pathway in diabetic contrast-induced acute kidney injury rats. We conclude that a signal pathway of pathophysiologic mechanism for apoptosis induced by iodinated contrast media (Figure 5A) and ERK1/2/JNK-caspase-3 mediated apoptosis pathway may be a target for probenol to prevent contrast-induced nephropathy (Figure 5B). Future experiments are needed to further characterize the apoptotic pathways involved in contrast-induced kidney injury, which would permit the discovery of novel therapeutic targets and preventive measures. However, because the present study had a small number of rats in each group, further verification of our results by studies with larger sample sizes is needed.

### Conclusions

Probenol can reverse the damaging effects of hypertonic contrast medium in the kidneys by promoting ERK1/2



**Figure 5.** The potential mechanism of apoptosis induced by iodinated contrast media and the effect of probenol on contrast-induced nephropathy.

phosphorylation, in turn leading to downregulation of JNK. This in turn altered the balance between Bcl-2 and Bax, leading to inhibition of mitochondrial caspase-3, thereby reducing renal cell apoptosis.

## Conflict of interest

None.

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