

Original Article

Combination of apigenin and ischemic postconditioning protects against renal ischemia/reperfusion injury in rat by inhibiting TLR4/NF- κ B signaling pathway

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Abstract: Purpose: To investigate the effect and possible mechanism of combination of apigenin and ischemic post conditioning on renal ischemia-reperfusion injury in rats. Materials and methods: Fifty rats were randomly separated into 5 groups: (1) sham-operation groups: a midline laparotomy was performed only; (2) I/R group: rats were underwent 45 min of renal ischemia; (3) apigenin group: rats was subjected to the same surgical procedures as I/R group, and apigenin was intravenously injected at 10 min prior to the experiment; (4) IPO group: I/R+three cycles of 10 secs of reperfusion and 10 secs of re-occlusion before full reperfusion; (5) apigenin+IPO group: IPO plus the apigenin pretreatment. Rats were sacrificed at 24 hours after I/R injury. Blood samples were collected for the detection of serum creatinine and urea nitrogen levels. Histologic examinations were evaluated. The expression of TLR4, NF- κ B, TNF- α , IL-1 β and ICAM-1 were performed by immunohistochemistry, Real-time PCR and Western blot. Results: Apigenin and IPO significantly reduced the increase of serum creatinine and urea nitrogen induced by renal ischemia/reperfusion, showing an improvement in renal function. The histologic evidence of renal damage associated with ischemia/reperfusion reduced by apigenin and IPO. Compared with I/R group, the expression of TLR4, NF- κ B, TNF- α , IL-1 β and ICAM-1 were downregulated by apigenin and IPO on mRNA and protein levels. Conclusions: The combination of apigenin and ischemic postconditioning inhibited TLR4/NF- κ B signal pathway in renal ischemia/reperfusion injury, provided remarkable protection against renal ischemia/reperfusion injury in rats.

Keywords: Combination, apigenin, ischemic postconditioning, TLR4, NF- κ B

Introduction

Renal ischemia/reperfusion injury (IRI) is a major cause of early acute renal failure in transplanted kidneys [1], which may lead to increased mortality rates and promote the succedent development of chronic kidney disease [2]. The reestablishment of blood flow is important to protect against ongoing injury. The previous existing ischemic damage would be aggravated by strengthening the inflammatory response [3]. Although reperfusion is essential for the survival of ischemic tissue, but earlier studies show that reperfusion itself causes additional injury [4]. There are many complex pathogenesis involved in renal ischemia/reperfusion injury. Acute renal failure is a predictor of standing graft viability. These pernicious effects are mediated by an intricate response to ischemia/

reperfusion involving damage-associated molecular mechanism, including oxygen radical species (ROS), chemokines, cytokines and autophagy [5]. The increased generation of reactive oxygen species and a decrease in endogenous anti-oxidant reserve can result in oxidant-induced damage. In addition, both oxidants and inflammatory mediators contribute to cell apoptosis and necrosis [6].

More and more research pay attention to organs protective strategies which creates resistance against organ ischemia and reperfusion injury [7]. Ischemic preconditioning (IP) and ischemic postconditioning (IPO) are two effective surgical methods to improve the resistance against renal ischemia/reperfusion injury [8, 9]. The clinical application of IP is mostly limited because IP must be launched before the IR and

the onset of an ischemic injury cannot be predicted. Unlike IP, unrestricted application of IPO is allowed in clinical settings, IPO is more clinically applicable and flexible, which can be used for the onset of reperfusion in the inchoate ischemic organ [10].

Apigenin is a plant flavone that exist in a variety of fruits and vegetables, such as celery, parsley and wheat sprout [11, 12]. Apigenin has been proven to possess a series of biological properties, such as anti-inflammatory, antioxidant, and antitumor effects [13]. Apigenin is a kind of antioxidant which had been reported to have protective effect on several organs. Apigenin can protect cells against apoptosis and necrosis by inhibiting oxidative stress. Recent studies showed that apigenin had a potent therapeutic effect on liver in rats [14]. However, there is no report about the effects of apigenin on renal ischemia/reperfusion injury. The purpose of our study was to investigate the protective role of apigenin and ischemic postconditioning against renal IRI in rats and the mechanism of this action, and to identify whether the underlying protective mechanisms are associated with the TLR4/NF- κ B-mediated apoptosis pathway in vivo.

Materials and methods

Drugs and reagents

Apigenin (Api, purity >98%) was purchased from Shanghai aladdin Biochemical Company. Anti-TLR4 and anti-NF- κ B antibodies were purchased from abclonal Company.

Animal preparation

This study was approved by the local ethical committee, and the experimental procedures were carried out in accordance with principles of Helsinki Declaration. Fifty adult male Sprague-Dawley rats (weighing 220 \pm 20 g) were provided by Hubei Provincial Academy of Preventive Medicine.

Experimental protocol

Fifty Sprague-Dawley rats were randomly separated into 5 groups: (1) sham-operation (n=10); (2) I/R (n=10); (3) apigenin (50 mg/kg) (n=10); (4) IPO (n=10); (5) apigenin+IPO (n=10). In the apigenin group and apigenin+IPO group, apigenin (50 mg/kg) was intravenously injected at 10 min prior to the experiment.

All the rats were anesthetized with chloral hydrate intraperitoneally (350 mg/kg). After i.v. Injection of heparin (1000 UI/kg), maintaining the body temperature at 37°C, a midline laparotomy was performed. In the I/R group, we performed a right nephrectomy, then we isolated the left renal pedicles (the artery, vein and nerve); The left kidney was subjected to 45 min of ischemia followed by reperfusion after right nephrectomy. Reperfusion launched the artery clips were removed. Change in the color of the kidneys to a paler shade and reperfusion by a blush verified occlusion. In sham operation group, rats were subjected to the same surgical procedures as I/R group without left renal clamping. In IPO group, rats were subjected to the same surgical procedures as I/R group, furthermore received renal intervention with three cycles of 10 seconds reperfusion and followed by 10 seconds re-occlusion before the onset of reperfusion reflow. At 24 h after I/R injury, all rats were killed. Blood samples (1 mL) were collected from the heart for the measurement of urea creatinine (Cr) and nitrogen (BUN). The left kidney was removed and fixed in 4% paraformaldehyde or immediately frozen, and stored at -80°C for routine paraffin embedding and different determinations.

Serum assays

Blood samples were centrifuged at 15000 g for 10 min and kept at -20°C until analyses. Serum was collected for assessing Cr and BUN.

Histological examination

Half of each kidney was removed and fixed in 4% paraformaldehyde, followed by routine paraffin embedding. According to the standard procedure, tissue sections were cut for 4 μ m thick and stained with HE for histologic grading. These morphological sections were assessed by an experienced renal pathologist. A grading scale sum up by paller's standard 18, was used for the histopathological assessment of renal ischemia-reperfusion injury.

Immunohistochemistry

The expression of TLR4 and NF- κ B were conducted by immunohistochemical staining. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide at 37°C for 10 min. Afterwards, these sections were treated with 1:50 normal horse serum to in Tris-buffered

Table 1. Effect of apigenin and IPO on the renal content of the BUN, Cr, at 24 h after reperfusion

Groups	Sham	I/R	Apigenin	IPO	Apigenin+IPO
BUN (mmol/L)	7.48±0.94	39.63±2.74*	26.27±2.04* [#] , [※]	22.83±1.83* [#] , [※]	17.63±1.74* [#]
Cr (μmol/L)	35.25±2.14	310.55±7.48*	207.65±6.36* [#] , [※]	220.64±6.73* [#] , [※]	180.32±5.87* [#]

The values are shown as means ± SEM (n=10). *P<0.01 versus sham; [#]P<0.05 versus I/R; [※]P<0.05 VS apigenin+IPO.

saline (TBS) for 30 min at 37°C. Then, rabbit anti-TLR4 antibody or rabbit anti-NF-κB antibody was applied respectively for incubating overnight at 4°C. PBS was used for washing these sections three times. After incubating with the secondary antibody for 30 min at 20°C, these sections were treated with color reagent DAB.

Realtime PCR

The extraction of total RNA in rat kidney was performed strictly by using TRizol RNA Reagent Kit (purchase from Takara, Japan). RNA reverse transcription into cDNA was performed following the instructions of the Applied Biosystems SYBR Green mix kit (purchase from Shanghai aladdin Biochemical Company). TNF-α forward primer 5'-GCCACCACGCTCTTCTGTC-3', and reverse primer 5'-GCTACGGGCTGTGCTACTCG-3'. IL-1β forward primer 5'-ACTATGGCAACTGTCCCTGAAC-3', and reverse primer 5'-GTGCTTGGGTCTCATCCTG-3'. ICAM-1 forward primer 5'-GGGATGGTGAAGTCTGTCAA-3', and reverse primer 5'-GGCGGTAATAGGTGTAATGG-3'. β-actin forward primer: 5'-TGCTATGTTGCCCTAGACTTCG-3' and reverse primer: 5'-GTTGGCATAGAGGTCTTTACGG-3'. 5 μL DNA Marker I was used as the control and 1.5% agarose gel electrophoresis was performed on 5 μL Rt-PCR product. Analysis of relative gene expression levels was performed by using β-actin as an endogenous reference gene.

Western blotting assay

The kidney tissues were dissociated by using the total protein extraction kit (purchase from Wuhan Goodbio technology company) according to the specification of the kit, and total proteins extracted were examined via Western blot. 40 μg weights of protein from each sample was separated on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. Then, the membranes were blocked with 5% non-fat milk in Tris-buffered saline and Tween 20 (TBST) buffer and incubated with primary polyclonal antibodies (1:500) of anti-TLR4 and anti-

NF-κB, and anti-GAPDH at 4°C overnight. After being washed triple with TBST for approximately 15 minutes, the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (1:2000 dilution). After that, the membranes were washed 3 times with TBS-T. Specific bands were visualized by using an enhanced chemiluminescence detection kit. The immune complexes were visualized by an enhanced chemiluminescence detection kit. Ultimately, the band intensity was detected using the Quantity One software.

Statistical analysis

All data were expressed as mean ± SD. The means of the different groups were compared using one-way ANOVA test. Differences were considered statistically significant when P<0.05 (*P<0.05).

Results

Combination of apigenin and IPO reduced postischemic renal dysfunction

As shown in **Table 1**. The renal functional parameters of rats of these groups were significantly different. Compared with sham-operated rats, the I/R group showed significant increases in BUN and Cr (P<0.01). And the renal function of rats treated with I/R was improved by treatment with apigenin and IPO (P<0.05). In addition, the BUN and Cr values in apigenin+IPO group were significantly lower than those in apigenin group and IPO group, which suggest that combination of apigenin and IPO would reduce postischemic renal dysfunction significantly.

Combination of apigenin and IPO improved the morphological features of injury

In I/R group, Morphologic abnormalities including tubular cell necrosis, cytoplasmic vacuolization and tubular lumen obstruction and impairments were found. Apigenin and IPO relieved these severe renal damages. The histologic scores by standard of paller in sham

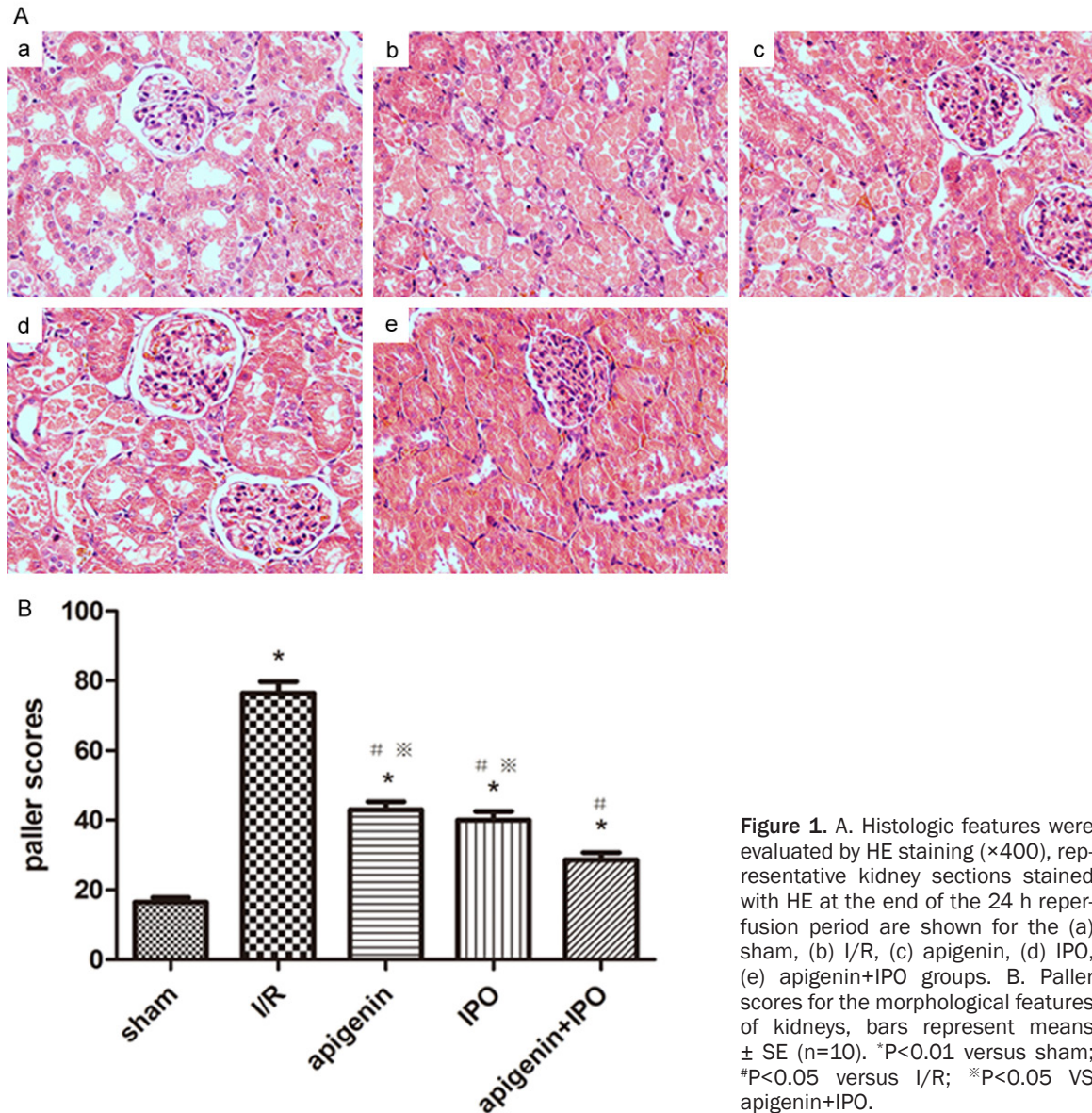


Figure 1. A. Histologic features were evaluated by HE staining (×400), representative kidney sections stained with HE at the end of the 24 h reperfusion period are shown for the (a) sham, (b) I/R, (c) apigenin, (d) IPO, (e) apigenin+IPO groups. B. Paller scores for the morphological features of kidneys, bars represent means ± SE (n=10). *P<0.01 versus sham; #P<0.05 versus I/R; ※P<0.05 VS apigenin+IPO.

group were observably lower than that in I/R group, and the scores dramatically decreased in apigenin group, IPO group and apigenin+IPO group compared with the I/R group. Furthermore, the scores in apigenin+IPO group were higher than which in the other two groups (Figure 1A, 1B).

Combination of apigenin and IPO inhibited the mRNA expression of TNF- α , IL-1 β and ICAM-1

To survey the difference of mRNA expression of TNF- α , IL-1 β and ICAM-1, the levels of TNF- α , IL-1 β and ICAM-1 were measured by Realtime-PCR. The expression of TNF- α , IL-1 β and ICAM-1

in kidney tissues at the level of mRNA showed significant increase in I/R group compared to sham-operated group (P<0.01). However, apigenin and IPO inhibited the mRNA expression of TNF- α , IL-1 β and ICAM-1 (P<0.05), in addition, the restraining levels of TNF- α , IL-1 β and ICAM-1 expression up to the highest by combination of apigenin and IPO (Figure 2).

Combination of apigenin and IPO inhibited the expression of TLR4 and NF- κ B

TLR4 and NF- κ B were localized by immunohistochemical techniques. The staining sections manifested that the expression of TLR4 and

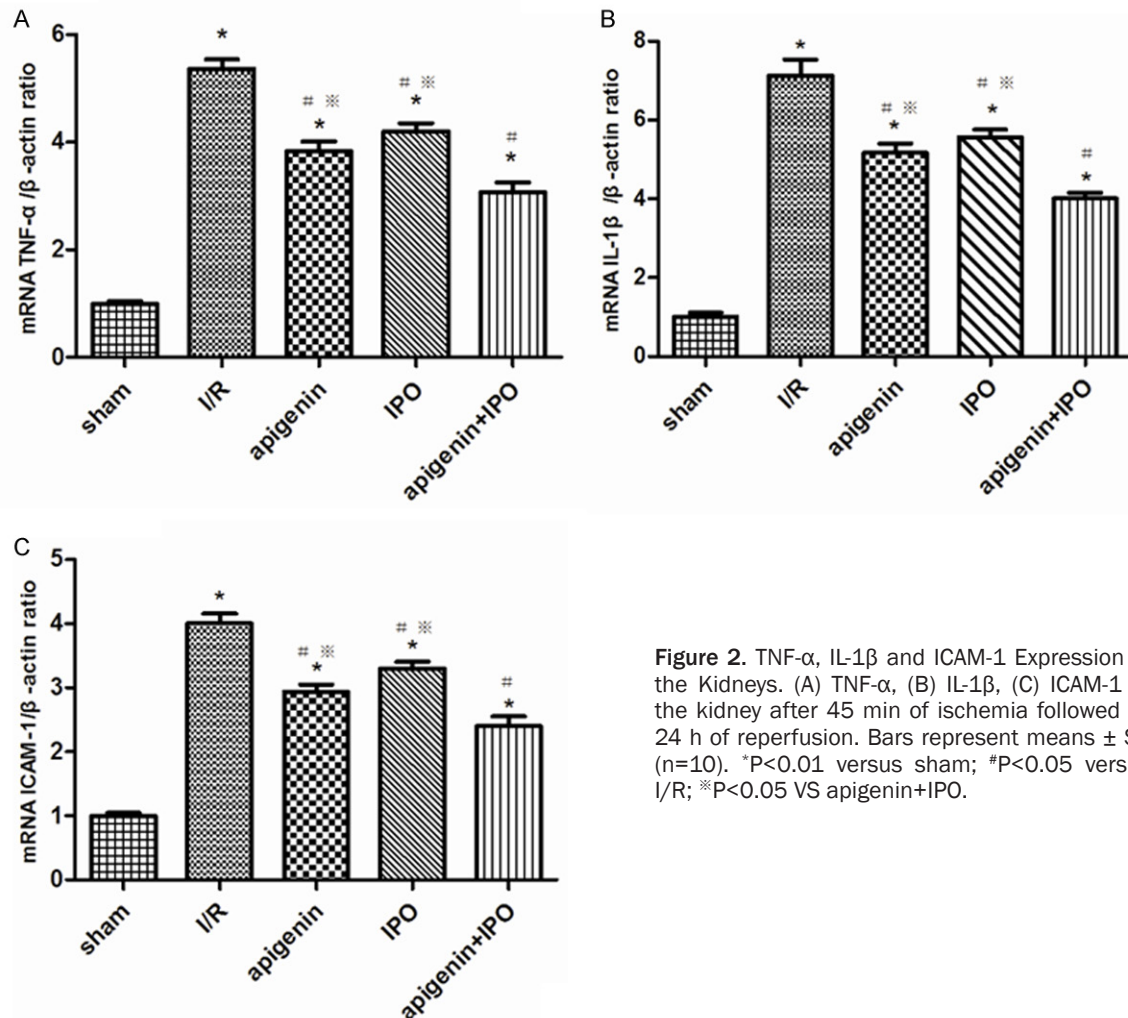


Figure 2. TNF- α , IL-1 β and ICAM-1 Expression in the Kidneys. (A) TNF- α , (B) IL-1 β , (C) ICAM-1 in the kidney after 45 min of ischemia followed by 24 h of reperfusion. Bars represent means \pm SE (n=10). *P<0.01 versus sham; #P<0.05 versus I/R; *P<0.05 VS apigenin+IPO.

NF- κ B proteins are localized in the cytoplasm of renal tubular cells. And renal tissues were greatly positive for TLR4 and NF- κ B expression in I/R group, this tendency was suppressed by apigenin and IPO treatment (**Figure 3A**). Analogously, these results were consistent with the Western blot analysis. Western blot analysis showed a significant increase of TLR4 and NF- κ B expression in I/R group compared with sham-operated group (P<0.01). The TLR4 and NF- κ B expression decreased in apigenin group, IPO group and apigenin+IPO group (P<0.05). As compared with the apigenin group and IPO group, the apigenin+IPO group induced the greater decrease of TLR4 and NF- κ B expression (**Figure 3B-D**).

Discussion

Renal ischemia/reperfusion injury results from a series of events. As reported, acute ischemia

induces ATP deficits and causes cell damage in kidney [15]. In the primary I/R injury phase, the inflammatory cascade responses is amplified, where after the inflammatory cells are activated, cytokine is secreted, and results in apoptosis or necrosis of the renal parenchyma cells ultimately [16]. Flavonoids are essential in many living plants, which can act as free radical scavengers and inhibitors of prooxidative enzymes to protect cells from oxidative damages and cancer [17]. Apigenin, a kind of flavonoids, exist in fruits, vegetables, tea, wine and coffee widely. Apigenin have been found to exert antimicrobial, antiviral, anti-inflammatory and anti-allergenic activities [18]. The antioxidant properties of apigenin can protect organisms from reactive oxygen species (ROS), inhibit the oxidative stress induced by Hypoxia-reoxygenation [19]. Results of its use in our present study are quite significant, which dem-

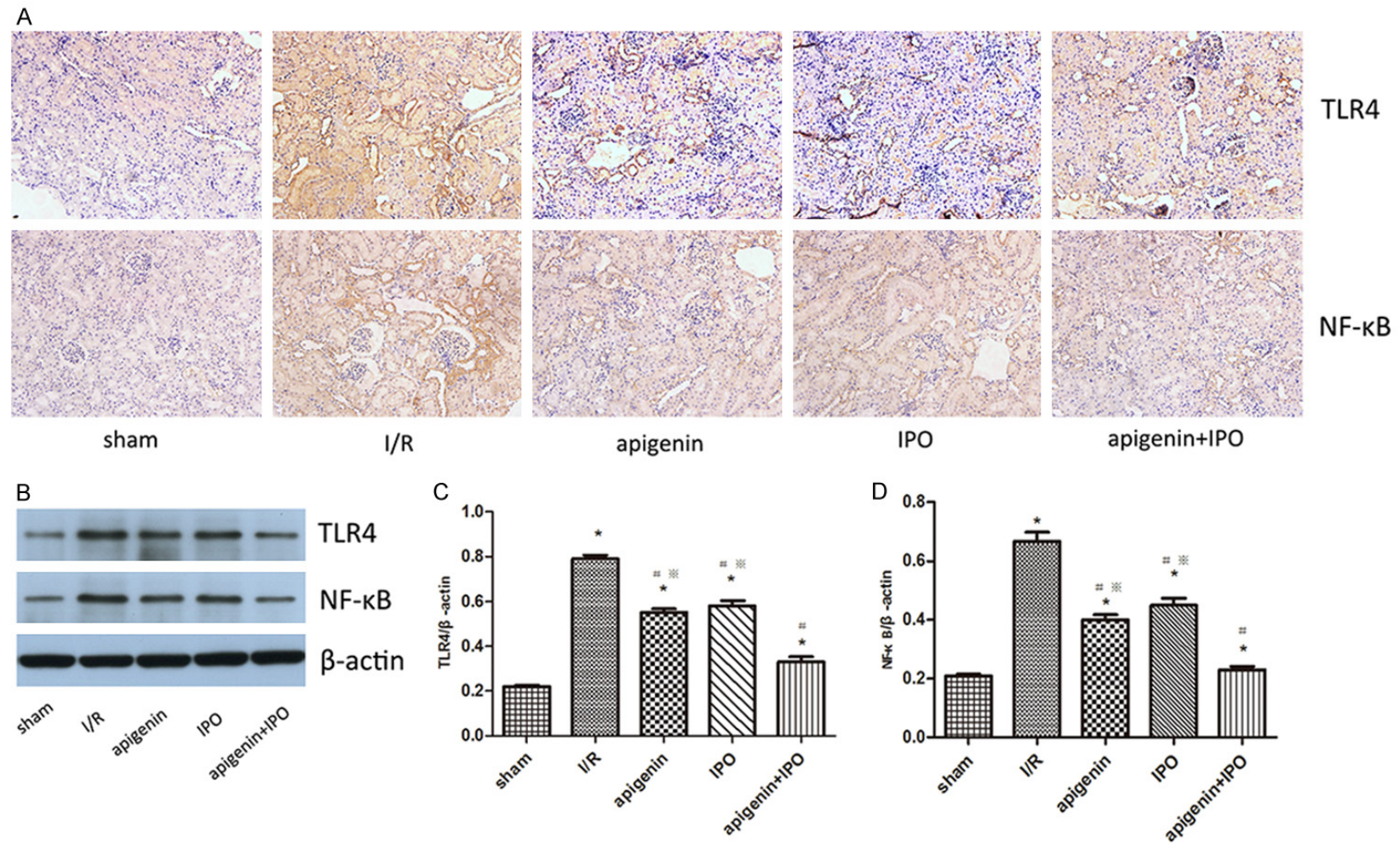


Figure 3. A. Immunohistochemistry was performed for expression of TLR4 and NF-κB. B. Representative western blot showing the expression of TLR4 and NF-κB in the kidney after 45 min of ischemia followed by 24 h of reperfusion. C. Quantitative analyses of the expression of TLR4. D. Quantitative analyses of the expression of NF-κB. Bars represent means \pm SE (n=10). *P<0.01 versus sham; #P<0.05 versus I/R; *P<0.05 VS apigenin+IPO.

onstrated that apigenin exerted significant protective effects against renal ischemia reperfusion injury in rats. IPO, first reported in 2003 by Zhao et al [20] as a cardioprotective strategy against cardiac I/R injury, is an effective technique described to alleviate ischemia reperfusion injury in brain, heart, liver and kidney. IPO is defined as rapid, desultory interruptions of blood flow in the early reperfusion phase [21]. Many studies have confirmed the renoprotection of IPO in the kidney [22-24], included our previous studies, but the explicit mechanisms are not fully understood.

In our experiment, we investigated whether the combination of apigenin and IPO produces a protective effect on the kidney suffering I/R injury by inhibiting TLR4/NF- κ B signaling pathway. We have been established renal ischemia-reperfusion injury rat model, and observed the change of expression of TLR4, NF- κ B and other inflammatory cytokines in rats. Toll-like receptor 4 (TLR4), a transmembrane protein, widely expressed on the tissue of the kidney, plays a important role in renal I/R injury [25]. TLR4 is a kind of the molecular mechanisms mediating the inflammatory effects in renal I/R injury. Many studies have suggested that the progression of renal I/R injury was promoted by TLR4 [26]. TLR4 could be combined with endogenous ligands, including damage-associated molecular patterns. Studies has shown that the inhibition of TLR4 genes in the renal alleviates renal I/R injury, downregulates TNF- α , IL-1 β and ICAM-1 expression and ameliorates I/R injury in renal tubular epithelial cells [27]. NF- κ B is a key transcription factor in TLR4-mediated signaling and plays a dominating role in promoting inflammatory responses induced by I/R (23). A lot of studies have shown that the absence of NF- κ B may lead to mitigation of I/R injury and the improvement of renal functional recovery, inhibition of chemokines and inflammatory cytokines expression [28]. In summary, inhibiting the TLR4/NF- κ B signaling pathway is a potential therapeutic target for protecting against renal ischemia/reperfusion injury. In our study, we investigated the TLR4 and NF- κ B expression at 24 hours after ischemia in the I/R group, apigenin group and IPO group. The results showed that apigenin and IPOs ignificantly inhibited the TLR4/NF- κ B signaling pathway and the inflammatory cytokines (TNF- α , IL-1 β and ICAM-1) in

renal I/R injury, that could be proved by immunohistochemistry, Real-time PCR and Western blot. The protections of apigenin and IPO may be derived from potent anti-inflammatory effect, such as inhibition of inflammatory cytokines function and tissue cytokine release.

The combination of apigenin and IPO was performed in our study, which is differed from the traditional therapeutic method of renal I/R injury. Clinical application of the traditional therapeutic method (treated with drugs only) is often restricted since the curative effect is not significant. A more clinically suitable approach is combined the pretreatment of apigenin with IPO which was performed at the onset of reperfusion. Our study further demonstrated the aforesaid findings, indicating that the combination of apigenin and IPO was able to reduce the levels of renal function and relieve severe renal damages caused by I/R injury. Furthermore, our study showed that I/R result in the prominent upregulation of the mRNA levels of TNF- α , IL-1 β and ICAM-1 and protein levels of TLR4/NF- κ B in nephridial tissue. However, this upregulation was especially inhibited by the combination of apigenin and IPO. These data indicated that the combination inhibit TLR4/NF- κ B signal pathway after renal I/R injury in rats. Moreover, the effect of combination is superior to the application of apigenin or IPO respectively.

There are some limits in our study. Many studies have demonstrated that IPO generated the renoprotection against renal IRI [29, 30]. However, the IPO strategy is inappropriate for other organs sometimes that is limited in clinical practice consequently. Compared with local IPO, Remote ischemic postconditioning (RIPO), which repeated several occlusion/release cycles at other tissues or organs, is a secure and feasible approach to provide organ protection to prevent IRI.

In conclusion, apigenin and IPO were proved to protect rats against inflammation response after renal I/R injury. The mechanism of combination of apigenin and IPO may be associated with the downregulation of TLR4 and NF- κ B expression. That is to say, inhibiting TLR4/NF- κ B signal pathway would decrease the nephropathy damages following renal I/R injury.

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Disclosure of conflict of interest

None.

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