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Original Research

Macelignan protects against renal ischemia-reperfusion injury via inhibition of inflammation and apoptosis of renal epithelial cells

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Abstract: Ischemia-reperfusion injury (IRI) refers to tissue damage that occurs when blood supply returns to tissue after a period of ischemia, anoxia or hypoxia. It occurs frequently during shock, organ transplantation and heart failure. It can cause impairment or even renal failure. Macelignan is a lignin isolated from the seeds of *Myristica fragrans*. It has been reported to inhibit neuroinflammation and oxidative toxicity. The preventive or therapeutic effects of macelignan on renal IRI has not been reported. The present study investigated the effects of macelignan on renal IRI in rats, and the underlying mechanism(s). Healthy adult male Sprague Dawley rats (n = 50) aged 7 – 9 weeks (mean weight = 220 ± 20 g) were used in this study. The rats were randomly assigned to five groups of 10 rats each: sham treated group, *IRI* group and 40 mg macelignan/kg body weight (bwt) group, 80 mg macelignan/kg bwt group, and 160 mg macelignan/kg bwt group. Ischemia-reperfusion injury was induced in the rats using standard procedure. The results showed that serum levels of creatinine, blood urea nitrogen (BUN), interleukin-6 (IL-6), tumor necrosis factor α (TNF-α) and gamma interferon (IFN-γ) were significantly higher in *IRI* group than in sham treated group, but were significantly and dose-dependently reduced after treatment with macelignan ($p < 0.05$). The activities of catalase and superoxide dismutase (SOD), and reduced glutathione (GSH) level were significantly reduced in *IRI* group, when compared with sham treated group, but were significantly and dose-dependently increased after treatment with macelignan ($p < 0.05$). However, the level of malondialdehyde (MDA) was significantly higher in *IRI* group than in sham treated group, but treatment with macelignan reduced it significantly and dose-dependently ($p < 0.05$). Macelignan also significantly and dose-dependently inhibited IRI-induced apoptosis in epithelial cells of renal tubules ($p < 0.05$). The results of Western blotting showed that IRI significantly upregulated the expressions of bax and caspase-3, and down-regulated the expression of bcl-2 in epithelial cells of renal tubules ($p < 0.05$). However, treatment with macelignan significantly and dose-dependently down-regulated the expressions of bax and caspase-3 in these cells, but significantly and dose-dependently upregulated the expression of bcl-2. These results show that macelignan confers protection on renal IRI via mechanisms involving inhibition of inflammation and apoptosis, and stimulation of natural antioxidant defense system.

Key words: Macelignan; Ischemia-reperfusion injury; Apoptosis; Kidney; Expression.

Introduction

Ischemia-reperfusion injury (IRI) or reoxygenation injury, refers to tissue damage that occurs when blood supply returns to tissue after a period of ischemia, anoxia or hypoxia (1). Renal IRI is the main cause of acute renal injury (ARI). It occurs often in kidney transplantation, nephrectomy, renal artery angioplasty, hydronephrosis, selective urinary tract surgery and post-shock resuscitation (2, 3). Renal IRI is a pathological process involving oxidative stress, inflammation, calcium overload and apoptosis. Apoptosis is recognized as the most important cause of IRI (4, 5). Renal IRI remains a major clinical problem, since there is yet no effective treatment for it (6). Salt corticosteroid receptor antagonists and dexmedetomidine have been reported to confer protection on renal IRI (7, 8). Nonetheless, it has become necessary to search for new drugs that can effectively treat renal IRI.

Myristica fragrans Houtt is an evergreen tree indigenous to the Moluccas of Indonesia. It is the main source of the spices, nutmeg and mace, and also possesses medicinal properties. It kernels and aril parts are used in folklore medicine. Its pharmacological effects include antibacterial, anti-inflammatory, antioxidant, anticancer, hepatoprotective, hypoglycemic and anti-hypertriacylglycerolemic and anti-hypercholesterolemic properties (9, 10).

Macelignan is a lignin isolated from the seeds of *M. fragrans*. It exerts antioxidant, anti-inflammatory and neuroprotective effects (11). Studies have shown that it reduces tert-butylhydroperoxide(t-BHP)-induced reactive oxygen species (ROS) production and DNA damage in cells (12). It also inhibits chronic ischemia or hypoxia-neuronal apoptosis in rats hippocampi. Macelignan has been reported to inhibit neuroinflammation and oxidative toxicity (13). However, the effects of macelignan on renal IRI has not been reported. The present study investigated the effects of macelignan on renal IRI in rats, and the underlying mechanism(s).

Materials and Methods

Drugs and reagents

Macelignan was obtained from MCE Corporation (USA). Gamma interferon (IFN-γ), IL-6, TNF-α en-

zyme-linked immunosorbent assay (ELISA) kits were purchased from Wuhan Huamei Biological Engineering Co. Ltd. TUNEL kit was purchased from Guangzhou Hengrui Biological Preparations Co. Ltd. Blood creatinine, BUN, catalase, SOD, GSH and MDA kits were products of Nanjing Institute of Bioengineering. Bicinchoninic acid (BCA) kit was purchased from Beijing Gede Biological Co. Ltd. Polyclonal antibodies of GAPDH, bcl-2, bax and caspase-3 were products of Santa Cruz Co. Ltd. (USA). Horseradish peroxidase (HRP)-labeled antibody was obtained from Shanghai Biyuntian Biotechnology Co. Ltd.

Experimental rats

Healthy adult male Sprague Dawley rats (n = 50) weighing 210 – 230 g (mean weight = 220 ± 20 g), and aged 7 - 9 weeks, were purchased from Shandong Laboratory Animal Center. They were housed in metal cages under standard conditions and had free access to standard feed and water. Prior to commencement of study, the rats were acclimatized to the laboratory environment for 3 days under 12 h light/12 h dark cycles and maintained at temperature of 24 ± 2 °C, and humidity of 40 – 70 %. The study protocol was approved by the Institutional Animal Care and Use Committee of the Animal Laboratory of Zhongshan Hospital of Traditional Chinese Medicine (TCM) Affiliated to Guangzhou University of TCM (No. SYXK 2015-0109). The study procedures were carried out according to the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Experimental design

The rats were randomly assigned to five groups of 10 rats each: sham treated group, *IRI* group and 40 mg macelignan/kg bwt group, 80 mg macelignan/kg bwt group, and 160 mg macelignan/kg bwt group. Ischemia-reperfusion injury was induced in the rats using standard procedure. One week before the establishment of rat model of IRI, rats in sham and *IRI* groups were administered normal saline once a day, while rats in the treatment groups received normal saline containing corresponding drugs once a day. Seven days later, renal IRI was established in the rats (14, 15). The rats were first fasted for 12 h before surgery and then administered 10 % chloral hydrate anesthesia (3.5 mL/kg bwt) intraperitoneally (i.p.). Kidney pedicle was located in the abdominal cavity through a 2 cm incision along the center of the abdomen. The kidney was quickly blocked on both sides with a non-invasive mini-artery clip. A change in kidney color from bright red to purple-black was an indication of a successfully established renal IRI. After 45 min, the kidney color was restored to bright red, an indication of restored blood perfusion, and the abdomen was subsequently closed up layer by layer and sutured. Rats in the sham treated group were operated the same way, but without blocking blood flow in renal pedicle. After operation, the rats were kept in conducive laboratory environment to properly recuperate with adequate water supply. The criteria for a successfully established rat model of renal IRI were: (1) change in kidney color to bright red after removing the clamp for 5 min; and (2) ability of rats to survive within 2 h after closing their abdominal cavities post-surgery.

Determination of levels of biochemical parameters

Twenty-four hoursafter renal IRI was successfully induced, abdominal aorta blood was collected and centrifuged at 4500 rpm for 10 min at 4°C to obtain serum. Serum levels of creatinine and BUN were determined using an automated biochemical analyzer. The levels of IL-6, TNF-α and IFN-γ were estimated using their respective ELISA kits.

Determination of activities of catalase and SOD, and levels of GSH and MDA in kidney homogenate

Portion of excised kidney was used to prepare 10 % tissue homogenate with normal saline. The homogenate was centrifuged at 3000 rpm for 10 min at 4°C to obtain supernatant. The activities of catalase and SOD, and levels of GSH and MDA were determined in the supernatant.

Apoptosis assay

Apoptosis was determined in epithelial cells of renal tubules using TUNEL assay. Portion of excised rat kidney was fixed with 4 % paraformaldehyde for 36 h, then washed with graded ethanol (100 – 50 %), distilled water and phosphate buffer, and embedded in paraffin. The paraffin-embedded tissue was sliced into bits (0.2 cm thickness) using refrigerated microtome, and dewaxed with xylene. Equal volume of ethanol and phosphate buffer was used to rinse tissue slices, which were then boiled with citrate buffer (pH 6.0). After cooling, the slices were rinsed again with phosphate buffer. The paraffin-embedded tissue sections were further processed according to instructions in TUNEL kit and then examined using fluorescence microscopy, observed and photographed, and the level of apoptosis was calculated.

Western blotting

The expressions of apoptosis-related proteins were determined in epithelial cells of renal tubules. Portions of excised kidney tissues were trypsinized and ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitor was used to lyse them for 30 min. The resultant lysate was centrifuged at 14000 rpm for 20 min at 4 °C, and the protein concentration of the supernatant was determined using BCA method. A portion of total cell protein (10 μg) from each sample was separated on 8 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 ° C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies for rabbit polyclonal anti-bax, caspase-3, bcl-2 and GAPDH, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with PBS and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Enhanced chemiluminescence (ECL). Respective protein expression levels were normalized to that of GAPDH which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD, and statistical analysis was performed using Graphpad Prism (7.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of macelignan on serum levels of creatinine and BUN

Serum levels of creatinine and BUN were significantly higher in *IRI* group than in sham treated group, but were significantly and dose-dependently reduced after treatment with macelignan ($p < 0.05$; Figure 1).

Effect of macelignan on serum levels of IL-6, TNF-α and IFN-γ in the rats

Serum levels of IL-6, TNF-α and IFN-γ were significantly higher in IRI group than in sham treated group, but were significantly and dose-dependently reduced after treatment with macelignan ($p < 0.05$). These results are shown in Figure 2.

Effect of macelignan on activities of antioxidant enzymes and levels of GSH and MDA in each group

As shown in Figure 3, the activities of catalase and SOD, and level of GSH were significantly reduced in *IRI* group, when compared with sham treated group, but were significantly and dose-dependently increased after treatment with macelignan ($p < 0.05$). However, the level of MDA was significantly higher in *IRI* group than in sham treated group, but treatment with macelignan reduced it significantly and dose-dependently ($p < 0.05$).

Effect of macelignan on the level of apoptosis in epithelial cells of renal tubules

Treatment with macelignan significantly and dose-dependently inhibited IRI- induced apoptosis in epithelial cells of renal tubules ($p < 0.05$; Figure 4).

Effect of macelignan on the expressions of apoptosis-related proteins in epithelial cells of renal tubules

The results of Western blotting showed that IRI significantly upregulated the expressions of bax and caspase-3, and down-regulated the expression of bcl-2 in epithelial cells of renal tubules ($p < 0.05$). However, treatment with macelignan significantly and dose-dependently down-regulated the expressions of bax and caspase-3, but significantly and dose-dependently upregulated the expression of bcl-2 ($p < 0.05$). These results are shown in Figure 5.

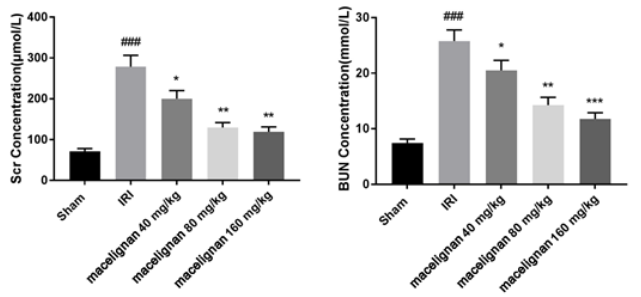

Figure 1. Effect of macelignan on serum levels of creatinine and BUN in rats. $^{\#}p < 0.05$, when compared with sham treated group; $^{*}p < 0.05$, $^{**}p < 0.01$ & $^{***}p < 0.001$, when compared with *IRI* group.

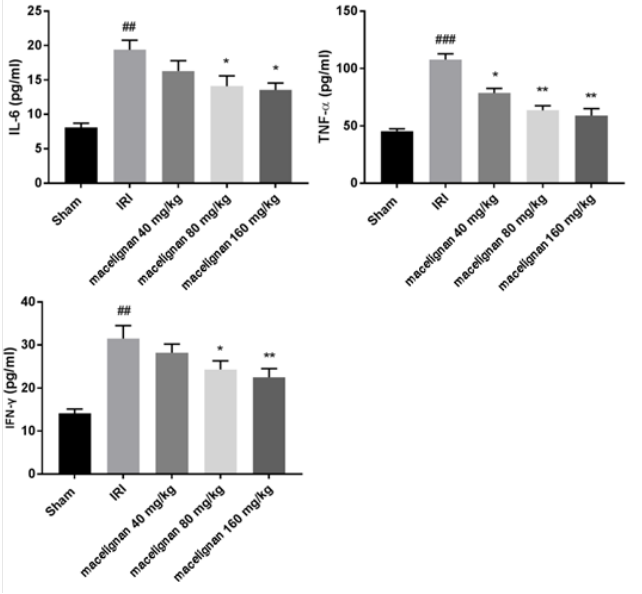

Figure 2. Effect of macelignan on serum levels of inflammatory markers in each group. $^{\#\#}p < 0.05$ & $^{\#\#\#}P < 0.01$, when compared with sham treated group. $^{*}p < 0.05$ & $^{**}p < 0.01$, when compared with *IRI* group.

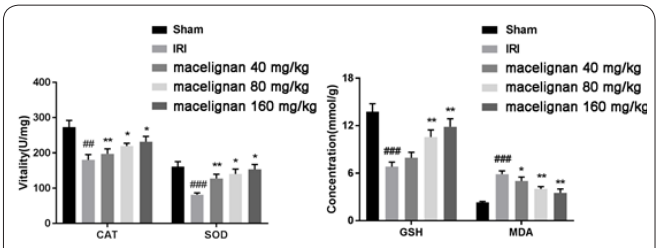

Figure 3. Effect of macelignan on activities of catalase and SOD, and levels of GSH and MDA in renal tissues of rats. $^{\#\#}p < 0.05$, $^{\#\#\#}p < 0.01$, when compared with sham treated group; $^{*}p < 0.05$ & $^{**}p < 0.01$, when compared with *IRI* group.

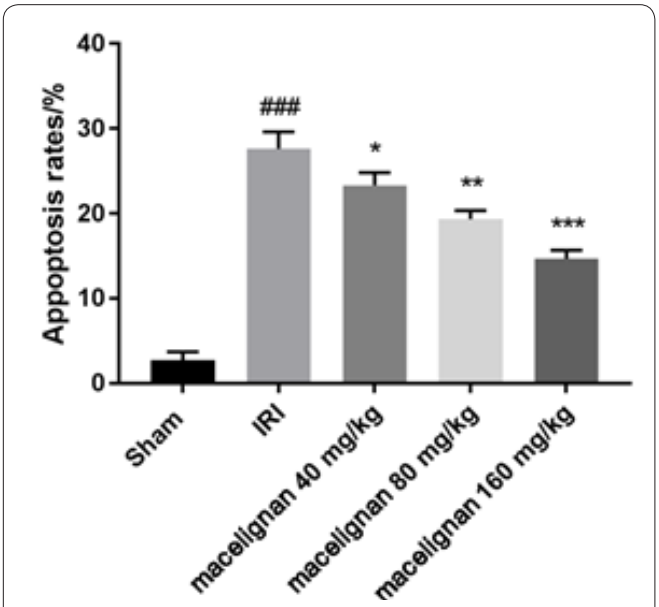

Figure 4. Effect of macelignan on level of apoptosis in epithelial cells of renal tubules in each group. $^{\#\#\#}p < 0.05$, when compared with sham treated group; $^{*}p < 0.05$, $^{**}p < 0.01$ &$^{***}p < 0.001$, when compared with *IRI* group.

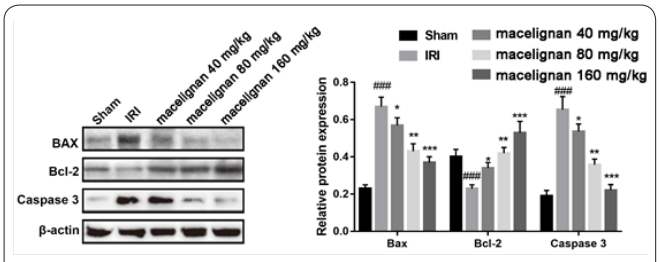

Figure 5. Effect of macelignan on bax, bcl-2, and caspase-3 protein expressions. $^{\#\#\#}p < 0.05$, when compared with sham treated group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, when compared with *IRI* group.

Discussion

The kidney is the most perfused organ in the body. It is highly sensitive to ischemia and IRI. The kidney is also highly susceptible to functional and metabolic disorders, once its blood supply is interrupted. Ischemia-reperfusion injury (IRI) is a common pathophysiological phenomenon which occurs during infection, shock, trauma, and kidney transplantation, and may lead to acute renal failure. The pathogenesis of IRI has not been fully elucidated, but studies have shown that it may be connected with the production of reactive oxygen species (ROS), decreased ATP level, increased intracellular calcium level, and infiltration and adhesion of inflammatory cytokines (16). The present study investigated the effects of macelignan on renal IRI in rats, and the underlying mechanism(s). Serum levels of creatinine and BUN directly reflect the state of glomerular filtration. In kidney injury, the levels of these markers are markedly increased, hence, they serve as sensitive indicators of renal function (17). In this study, serum levels of creatinine and BUN were significantly higher in *IRI* group than in sham treated group, but were significantly and dose-dependently reduced after treatment with macelignan. These results suggest that IRI-induced renal damage may be effectively repaired by macelignan.

In recent times, the connection between inflammation and renal IRI has received a lot of attention (18). The levels of expression of IL-6, TNF-α and IFN-γ reflect the level of inflammation and degree of tissue damage, and their serum levels increase with the intensity of inflammation (19). The results of this study suggest that macelignan may effectively reduce inflammatory injury by reducing the levels of these inflammatory factors. Similarly, studies have shown that oxidative stress is an important pathological factor that can lead to renal IRI (20). At the stage of renal ischemia, the degradation of AMP leads to accumulation of hypoxanthine. Restoration of renal blood flow and perfusion results in the production of a large number of ROS. Elevated levels of ROS and their degradation products lead to peroxidation of mitochondrial membranes, and ultimately renal damage. Malondialdehyde (MDA) is the end product of lipid peroxidation. Alterations in MDA levels may reflect the intensity of oxidative stress and the degree of tissue damage. The activities of catalase and SOD, and level of GSH reflect the ability of cells to effectively mop-up ROS and reduce oxidative stress (21).

The results of this study showed that the activities of catalase and SOD, and level of GSH were significantly reduced in *IRI* group, when compared with sham treated group, but were significantly and dose-dependently increased after treatment with macelignan. However, the level of MDA was significantly higher in *IRI* group than in sham treated group, but treatment with macelignan reduced it significantly and dose-dependently. These results suggest that macelignan may protect renal tissue against IRI by reducing oxidative stress. It is likely that pretreatment with macelignan may enhance the ability of kidney nephrons to scavenge free radicals and reduce intracellular levels of lipid peroxides.

It is generally believed that apoptosis of renal tubular cells is involved in renal IRI (22). Studies centered on caspase-3 activity, bax activation, cytochrome c release and changes in bcl-2/bax have provided evidence in support of the speculation that apoptosis causes IRI (23). Caspase-3, an important cysteine protease is synthesized as an inactive proenzyme or zymogen. This protein which is located downstream in the apoptotic pathway, plays a key role in apoptosis (24). In this study, IRI significantly upregulated the expressions of bax and caspase-3, and down-regulated the expression of bcl-2 in epithelial cells of renal tubules. However, treatment with macelignan significantly and dose-dependently down-regulated the expressions of bax and caspase-3, but significantly and dose-dependently upregulated the expression of bcl-2. These results indicate that macelignan may influence apoptosis via down-regulation of caspase-3 protein expression.

The results obtained in this study have shown that macelignan confers protection on renal IRI via mechanisms involving inhibition of inflammation and apoptosis, and stimulation of natural antioxidant defense system.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jianjun Li; Jianhua Long, Kun Qian, Shubo Tan, Jia Liu, Jianjun Li collected and analysed the data; Jianhua Long wrote the text and all authors have read and approved the text prior to publication.

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