European Review for Medical and Pharmacological Sciences 2020; 24: 12380-12388

Hydroxytyrosol inhibits apoptosis in ischemia/reperfusion-induced acute kidney injury *via* activating Sonic Hedgehog signaling pathway

Q. FANG, Y. ZHANG, D.-S. JIANG, Y. CHEN

Department of Nephrology, Taizhou People's Hospital, The Fifth Affiliated Hospital of Nantong University, Taizhou, China.

Abstract. – OBJECTIVE: Acute kidney injury (AKI) is a common critical illness in clinic, which seriously threatens the life of patients. The aim of this study was to validate the anti-apoptotic effect of hydroxytyrosol (HT) in ischemia/reperfusion (I/R)-induced AKI.

MATERIALS AND METHODS: The cell model of AKI was established by hypoxia/reoxygenation (H/R), and the animal model of AKI was established by I/R. The apoptosis was observed by Caspase-3 activity assay, flow cytometry and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining. Cell viability was detected by cell counting kit-8 (CCK-8) assay. Protein expression was measured by Western blot and mRNA level was analyzed by quantitative real-time polymerase chain reaction (RT-PCR). Renal function was assessed by measuring serum creatinine (Cr) and blood urea nitrogen (BUN).

RESULTS: H/R induced apoptosis of HK-2 cells and reduced cell viability. When HK-2 cells were pretreated with HT, apoptosis was markedly inhibited, and cell viability was greatly increased. In addition, HT could inhibit I/R-induced apoptosis of rat kidney cells and could notably improve rat kidney function. H/R promoted Sonic Hedgehog (SHH) expression in HK-2 cells, while HT treatment further enhanced SHH expression. Similarly, I/R induces SHH expression in kidney tissue, and HT could further promote SHH expression.

CONCLUSIONS: These results indicated that HT could inhibit apoptosis in I/R-induced AKI via activating SHH signaling pathway.

Key Words:
Hydroxytyrosol, Ischemia/reperfusion, Acute kidney injury, Sonic Hegdehog signaling pathway, Apoptosis.

Introduction

As a common clinical syndrome, acute kidney injury (AKI) is characterized by acute decline in renal function, with high morbidity and mortality, and its harm to public health around the world has also attracted more and more attention[1,2]. Although considerable progress has been made in basic scientific research, medical updates, and renal replacement therapy in the past decade, including epidemiology and large-scale database research, the search for new noninvasive biomarkers, the update of AKI guidelines and consensus of relevant experts, and the discussion of suitable renal replacement treatment methods, the average mortality rate of AKI is still as high as 14% to 60%, and the mortality rate of AKI among critically ill patients is 50% to 80%[3-5]. AKI was once regarded as a benign and reversible syndrome. Previously, it was believed that patients with AKI could be reversed by actively treating their kidney damage after the onset, but now it is found that many patients with AKI have irreversible kidney damage. It will progress to chronic kidney disease (CKD) and even end-stage renal disease (ESRD).

Ischemia/reperfusion (I/R) is one of the most important causes of AKI. It is commonly seen in clinical situations such as hemorrhagic shock, heart surgery, and kidney transplantation. Renal IRI refers to the phenomenon that ischemic kidney tissue is further aggravated after the blood is restored and it is a very complicated pathological process. At present, people do not fully understand it. During cardiac surgery, the kidney undergoes temporary ischemia and, then, blood flow recovery occurs, which further aggravates the ischemic injury. This pathophysiological process belongs to IRI. AKI caused by IRI is a serious complication after cardiopulmonary bypass, which can increase short-term mortality and prolong hospital stay. AKI caused by IRI is an independent risk factor for increased mortality within 10 years after extracorporeal circulation, and even short-term decline in renal function after

Corresponding Author: Yan Chen, MM; e-mail: cy2200@stu.just.edu.cn

surgery can become an independent risk factor for progression to CKD within 5 years[6-9]. In addition to cardiac surgery, I/R is also common in kidney transplantation, which involves transient ischemia and hypoxia of the donor kidney and renal blood flow reperfusion after transplantation into the recipient. IRI is inevitable in this process and is also a risk factor for AKI, delayed recovery of transplanted kidney function, and chronic rejection[10-12]. In view of the high morbidity and mortality of AKI caused by IRI and the lack of specific treatment measures, we need to further explore its pathophysiological mechanism to lay a solid foundation for the discovery of potential treatment methods in the future.

Hydroxytyrosol (HT), also known as 3,4-dihydroxyphenylethanol, is a phenolic compound in virgin olive oil of the Mediterranean diet, which has strong antioxidant activity and various physiological and pharmacological functions[13]. In recent years, many studies have indicated various biological effects of HT, including antioxidant, anti-inflammatory, blood lipid regulation, protection of the cardiovascular system, protection of the nervous system, immune regulation and anti-tumor biological activities[14-17]. Pan et al[18] found that HT could protect liver from IRI through anti-oxidative stress. Pei et al[19] demonstrated that HT can inhibit myocardial apoptosis induced by I/R. However, the role of HT in I/R-induced AKI is unknown. In this paper, we established *in vivo* and *in vitro* model of AKI induced by I/R to study the role of HT in it. The findings in this article provide a potential treatment for I/R-induced AKI.

Materials and Methods

Cell Culture

We used Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12) (Gibco, Rockville, MD, USA) complemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 1% penicillin / streptomycin (Gibco, Rockville, MD, USA) to culture HK-2 cells (Procell, Wuhan, China) in the cell incubator containing 5% CO_2 at 37°C. HK-2 cells were treated with H/R to construct I/R-induced AKI model, specifically: HK-2 cells were placed in an hypoxic incubator for 12 hours and then placed in normoxic incubator for 2 hours. The cells were divided into 3 groups: control group, H/R group, and H/R+HT group. The cells in the H/R+HT group were pretreated with HT for 12 hours before H/R treatment.

Establishment of I/R-Induced AKI Model

All 30 male Sprague-Dawley (SD) rats were purchased from the Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). All animal experiments were approved by the Animal Research Committee of Taizhou People's Hospital and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental animal feeding environment is SPF level, the environmental humidity is constant at about 55%, the temperature is constant at about 24°C, and the illumination mode adopts a 12-hour dark/12-hour illumination system. The experimental animals were housed in cages (4-5 animals per cage). Drinking water and feed were adequately given every day, and the litter was changed every 3 days. When the experimental animals are used in the experiment, the body weight is controlled at 200-250 g and the age is 7-9 weeks. The experimental animals were used in the experiment after acclimating for one week in the experiment center. The rats were fasted for 12 hours before surgery and had free access to water. After the rats were weighed, 2% pentobarbital was injected intraperitoneally at a dose of 50 mg/kg. Whether the rat was under anesthesia was determined by the following methods: decreased muscle tone, no pain reflex, and righting reflex disappeared. It is necessary to pay attention to the state of the rat at any time during the operation. After the rats were successfully anesthetized, we fixed the rats in a supine position, prepared the operation area with a shaving knife, sterilized the skin with iodophor, and laid a sterile drape. After carefully cutting the skin with the surgical scissors in the middle of the abdomen, the rectus abdominis and peritoneum were separated layer by layer to expose the abdominal cavity. After that, we gently pulled out the intestine and covered it with sterile gauze moistened with warm saline. After exposing the kidneys on both sides, we separated the surrounding tissue of the kidney pedicles to expose the kidney pedicles. The renal pedicles of both sides were clamped with vascular clips, and the kidney was observed to change from bright red to dark purple due to ischemia. Afterwards, the abdominal wall structure was carefully tightened, and the wound was covered with sterile gauze, and the rat was placed at a 37°C constant temperature heating plate. After 30 minutes, the kidney was exposed again, the blood vessel clip was loosened, and the kidney returned to bright red within 5 minutes, indicating

that the kidney resumed perfusion, and the I/R-induced AKI model was successfully constructed. Finally, we sutured the muscles, fascia, and skin layer by layer. After the rats were awakened from anesthesia, the rats were returned to the feeding cage, and normal diet and drinking water were restored. The rats were randomly divided into three groups: sham operation group, I/R group, and I/R+HT group. In the sham group, the renal pedicle was freed after opening the abdominal cavity without clamping. Rats in the I/R+HT group were intraperitoneally injected with HT before surgery.

Cell Counting Kit-8 (CCK-8) Assay

We placed HK-2 cells in a 96-well plate. The viability of cells was examined using CCK-8 reagent (10 μL) (MCE, Nanjing, China) to explore the effect of HT on HK-2 cells. The absorbance at 450nm was detected using a microplate reader.

Western Blot

The total protein was extracted by a protein extraction kit (Camilo Biological, Nanjing, China). We measured the protein concentration by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Then we added the loading buffer to the total protein and boiled the mixture for about 10 minutes. After that, we took the same amount of total protein and performed electrophoresis with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Then, we transferred the protein to the polyvinylidene difluoride (PVDF) (EpiZyme, Shanghai, China) membrane. 5% concentration of skim milk was prepared with TBST to block the non-specific antigens of the protein. After 2 hours, the primary antibodies were used to incubate the membranes overnight (Bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Bax, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; SHH, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Abcam, Cambridge, MA, USA, Rabbit, 1:1000), followed by incubation with secondary antibodies for 1.5 hours. After the membranes were washed 3 times for 30 minutes, the blots were exposed by Image Lab™ Software.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

TRIzol reagent (MCE, Nanjing, China) was utilized to extract the total RNA. Then, chloroform was added in 1/5 times of the amount of TRIzol. We let the Eppendorf (EP) tubes shake and then let them stand for 5 minutes. After that, the EP tubes were centrifuged with a centrifugal force of 12000 g for 20 minutes at 4°C. We then aspirated the upper aqueous phase of the mixture obtained by centrifugation and added an equal amount of isopropanol, mixed them and placed the mixture at 4°C for 15 minutes. Then, we centrifuged the mixture at 4°C for 15 minutes with a centrifugal force of 12000 g, after which the supernatant was discarded, and 1 ml of a 75% ethanol solution was added. The solution was centrifuged at 4°C for 10 minutes with a centrifugal force of 7500 g, after which the supernatant was discarded, dried at room temperature, and 20 μL of ribonuclease free water was added. Finally, we used NanoDrop™ 8000 to measure RNA concentration.

Reverse transcription was performed using reverse transcriptase kit (MCE, Nanjing, China). Real-time PCR was performed using Prism 7900 System. We used a 10 μL reaction system in accordance with the protocol. GAPDH was used to standardize the data. All the primers were listed in Table I.

Flow Cytometry

HK-2 cells were treated as described above. The supernatant was then collected, and adherent cells were collected after digestion with trypsin. We centrifuged the cell suspension with a centrifugal force of 200 g for 5 minutes, discarded the supernatant, washed with PBS, and centrifuged again in the same manner, repeating twice. Then, we resuspended the cells with 100 μL of Bind-

Table I. Real time PCR primers

Gene name	Forward (5′>3′)	Reverse (5′>3′)
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
SHH	CCTGTCTGGGTGGGGAT	GTGTGCGCTCCTCCTTG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative reverse-transcription polymerase chain reaction.

ing Buffer. After that, we added 5 μL of Annexin V-FITc (KeyGen, Shanghai, China) and 5 μL of propidium iodide (PI) (KeyGen, Shanghai, China) in per tube. Finally, the apoptotic cells were detected using flow cytometry.

TUNEL Staining

One Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) was used as instructed by the manufacturer to detect the apoptotic cells. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China).

Caspase-3 Activity

Caspase-3 activity of kidney tissue was detected using Caspase-3 activity detection kit (Beyotime, Shanghai, China).

Detection of Serum Cr and BUN

The serum Cr or BUN were detected using serum Cr assay kit or BUN assay kit (BioAssay Systems, Hayward, CA, USA).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were represented as mean ± Standard Deviation (SD). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way analysis of variance (ANOVA) test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

Toxicity and Protection of HT on HK-2 cells

To explore the optimal concentration of HT to treat HK-2 cells, we used different concentrations (100, 200, 300, 400, 500, 600 μM) of HT to treat HK-2 cells. It can be found from Figure 1A that when the concentration of HT exceeds 500 μM, HK-2 cell viability begins to decrease significantly. Afterwards, in order to continue to study the concentration of HT for the best protection of HK-2, HK-2 cells were pretreated with different concentrations (100, 200, 300, 400 μM) of HT, followed by H/R treatment, then the cell viability was detected. When the concentration of HT was 200 μM, the cell viability reached the maximum (Figure 1B). In summary, we chose 200 μM of HT for subsequent experiments.

HT Inhibited H/R-Induced Apoptosis in HK-2 cells

We examined the expression of Bcl-2 and Bax proteins by Western blot (Figure 2A). It can be seen that Bcl-2 expression in the H/R group was greatly decreased compared with the control group, while the level of Bax was greatly increased. After using HT, the level of Bcl-2 was increased and the level of Bax was decreased (Figure 2B and 2C). Similarly, we detected the levels of Bcl-2 and Bax mRNA using RT-PCR, which was consistent with the results of Western blot (Figure 2D and 2E). To further demonstrate the anti-apoptotic effect of HT, we used flow cytome-

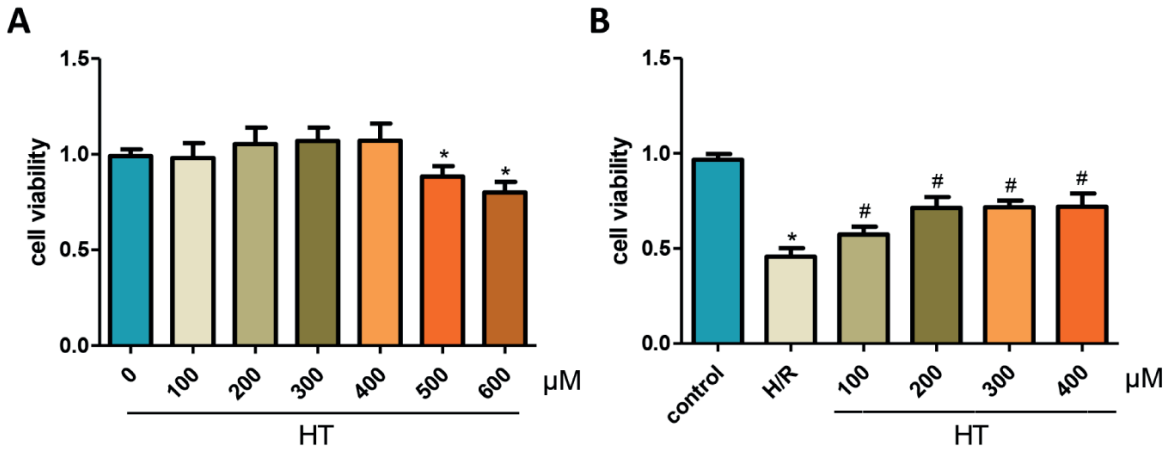

Figure 1. Toxicity and protection of HT on HK-2 cells. **A,** The cell viability was detected by CCK-8 assay ("$_*$" $p<0.05$ *vs.* 0, n=3). **B,** The cell viability was detected by CCK-8 assay ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H/R, n=3).

try to detect the rate of apoptotic cells. We can see that HT can markedly reduce the apoptotic rate of HK-2 cells treated with H/R (Figure 2F). We also used TUNEL staining to detect the effect of HT. It can be seen that there were fewer TUNEL-positive cells in the H/R+HT group than in the H/R group (Figure 2G and 2H). These results indicated that HT can inhibit H/R-induced apoptosis in HK-2 cells.

HT Reduced I/R-Induced Apoptosis of Kidney Tissue In Vivo and Improved Kidney Function

We tested rat serum Cr and BUN to assess rat kidney function. Compared with the sham group, serum Cr and BUN levels were notably increased in the I/R group. While compared with the I/R group, the serum Cr and BUN levels in the I/R+HT group were significantlly reduced

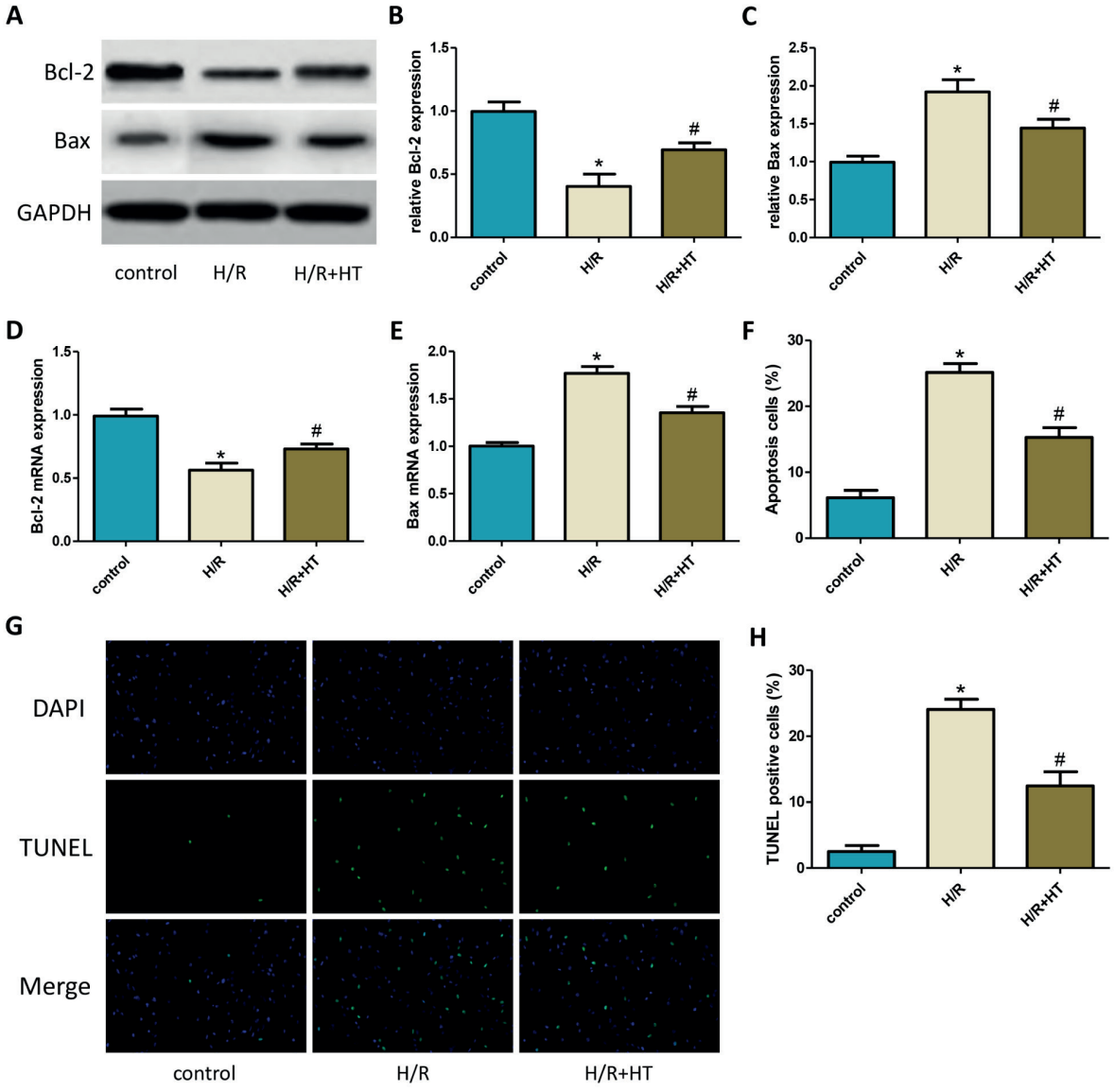

Figure 2. HT inhibited H/R-induced apoptosis in HK-2 cells. **A,** Western blot analysis of Bcl-2 and Bax. **B,** Statistical result of expression of Bcl-2 ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3). **C,** Statistical result of expression of Bax ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3). **D,** RT-PCR analysis of Bcl-2 mRNA ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3). **E,** RT-PCR analysis of Bax mRNA ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3). **F,** Apoptosis rate was detected by flow cytometry ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3). **G,** Results of TUNEL staining (magnification: 200×). **H,** Statistical result of TUNEL positive cells ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3).

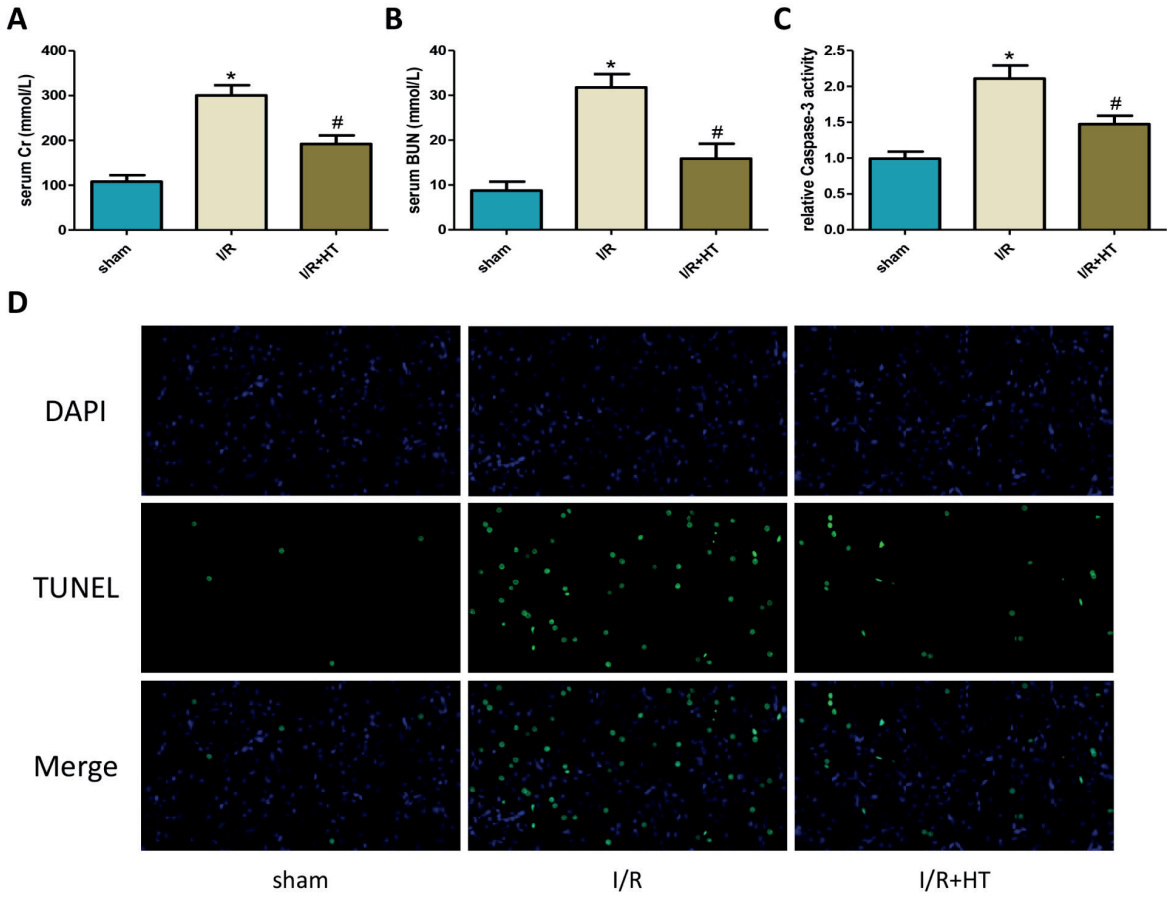

Figure 3. HT reduced I/R-induced apoptosis of kidney tissue *in vivo* and improved kidney function. **A,** and **B,** The levels of serum Cr and BUN were detected using commercial kits ("$_{*}$" $p<0.05$ *vs.* sham, "$_{\#}$" $p<0.05$ *vs.* I/R, n=6). **C,** The activity of Caspase-3 was detected using Caspase-3 activity detection kit ("$_{*}$" $p<0.05$ *vs.* sham, "$_{\#}$" $p<0.05$ *vs.* I/R, n=6). **D,** Results of TUNEL staining of kidney tissues (magnification: 400×).

(Figure 3A and 3B). We also tested the activity of Caspase-3 in kidney tissue and found that HT can remarkably inhibit the increase of Caspase-3 activity induced by I/R (Figure 3C). In addition, the results of TUNEL staining of renal tissue suggested that HT could reduce I/R-induced apoptosis (Figure 3D).

HT Activated the SHH Signaling Pathway

We detect the expression of SHH pathway *in vivo* and *in vitro* by detecting the levels of SHH protein and mRNA. We found that in HK-2 cells, H/R treatment induced SHH expression, while HT further increased SHH expression (Figure 4A-4C). Similarly, I/R increased SHH expression in the kidney, and HT can further induce SHH expression (Figure 4D-4F). These indicated that HT could activate the SHH signaling pathway.

Discussion

The kidney is a highly perfused organ and is very sensitive to ischemia. Renal vascular surgery, kidney transplantation, cardiac arrest, and hypotension shock can all cause kidney IRI to varying degrees. In recent years, with the increase in kidney transplantation and major cardiac surgery, the incidence and mortality of renal I/R have also increased year by year, and the mortality rate accounts for 20% of hospitalized patients[20]. Therefore, further research on the occurrence of renal IRI and the body's reaction mechanism is of great significance to understand the occurrence and development of renal IRI and take effective prevention measures early.

In the AKI induced by I/R, the characteristic histological changes of renal tubular cells include

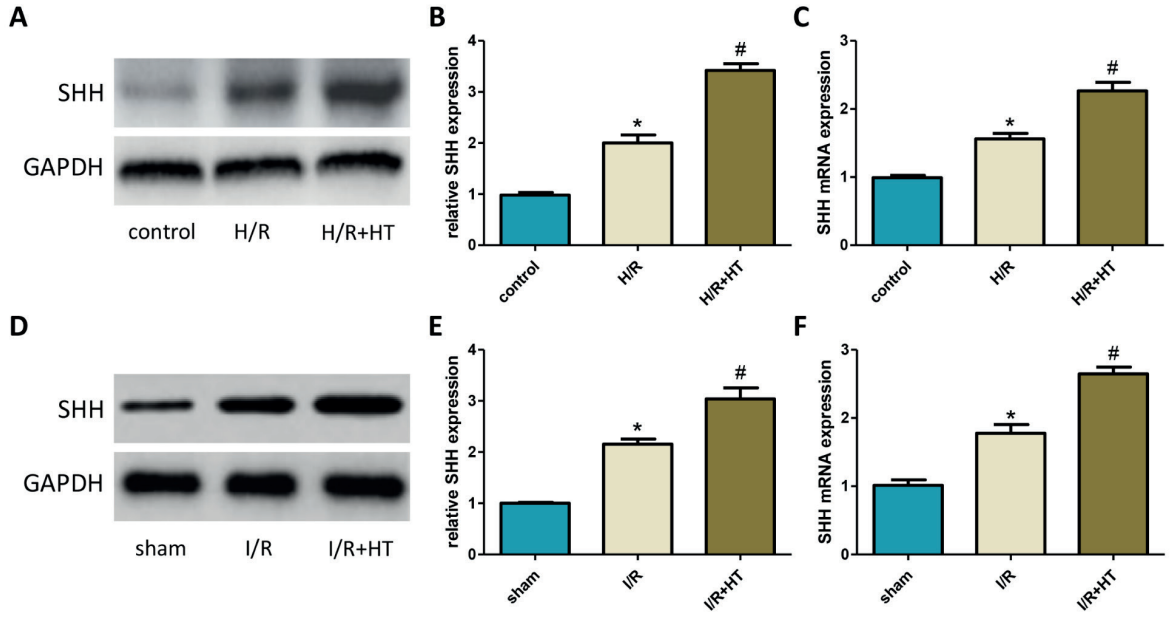

Figure 4. HT activated the SHH signaling pathway. **A,** Western blot analysis of SHH. **B,** Statistical result of expression of SHH ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3). **C,** RT-PCR analysis of SHH mRNA ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H/R, n=3). **D,** Western blot analysis of SHH. **E,** Statistical result of expression of SHH ("$_{*}$" $p<0.05$ *vs.* sham, "$_{\#}$" $p<0.05$ *vs.* I/R, n=3). **F,** RT-PCR analysis of SHH mRNA ("$_{*}$" $p<0.05$ *vs.* sham, "$_{\#}$" $p<0.05$ *vs.* I/R, n=3).

the loss of tubular brush border, the shedding of tubular epithelial cells or the fragmentation of necrotic cells into the tubular cavity, the expansion of the tubular cavity, and the formation of tubular castings caused by necrosis and apoptosis[21]. So, apoptosis may be the main mode of injury. Apoptosis is regulated by a variety of factors, and the combined effects of apoptosis-inducing and anti-apoptotic genes determine the fate of the cell. Both Bcl-2 and Bax belong to the Bcl-2 gene family. Bcl-2 is an apoptosis inhibitory gene, and Bax is a pro-apoptotic gene, both of which work together to regulate cell apoptosis. Sun et al[22] showed that the ratio of Bcl-2/Bax decreased after IRI in rat kidneys, which led to increased apoptosis of renal tubular epithelial cells. Caspases family is cysteine protein lyase, which is an important signal transduction pathway in the process of apoptosis. Among them, Caspase-3 is an important cysteine protease in the form of inactive zymogen, which is located downstream of apoptosis and is the only way for various apoptosis pathways. Zheng et al[23] found in the mouse kidney IRI model that the expression of Caspase-8 and Caspase-3 in the ischemic kidney was significantly increased. Moreover, Caspase-3 can cleave Bcl-2, and the cleavage product can further activate downstream Caspase, and lead to amplification of the Caspase cascade reaction, further promoting apoptosis[24].

In this study, in the H/R-induced HK-2 cell damage model, we observed that Bcl-2 expression decreased and Bax expression increased, and the apoptosis rate increased significantly. In addition, in the rat AKI model induced by I/R, Caspase-3 activity increased and accompanied by an increase in apoptosis rate. These suggest that apoptosis plays an important role in IRI. However, after treatment with HT, H/R-induced apoptosis of HK-2 cells was significantly inhibited, and the apoptosis of rat kidneys was also markedly reduced. Further, kidney function was also improved. In addition, we detected the SHH pathway by Western blot and found that the SHH pathway was activated in I/R-induced AKI, and after treatment with HT, the SHH pathway was further activated.

SHH is a highly conserved signaling pathway that is ubiquitous in the animal kingdom and plays an important role in embryonic development, tissue differentiation, organ formation, and maintaining the steady state of adult tissues. In the normal kidney tissue that has completed development, the SHH signaling pathway is in a low activity state, but in the case of I/R, obstruction, drug toxicity, etc., the signaling pathway can be

activated again. SHH signaling pathway has been found to be involved in the protection against renal IRI. Meng et al[25] found that polydatin can reduce oxidative stress and apoptosis induced by renal IRI by activating the SHH pathway. In addition, in the early stage of kidney injury, the SHH pathway can promote kidney fibrosis to participate in kidney protection and improve kidney function[26,27]. In this study, we found that I/R can activate the SHH pathway in the kidney, and HT treatment can further promote the activation of this pathway.

Conclusions

The present results indicated that the apoptosis of renal tubular epithelial cells is involved in the process of renal IRI, and revealed for the first time that HT reduces renal IRI by inhibiting the apoptosis of renal tubular epithelial cells. In addition, the protective effect of HT on the kidneys was achieved by activating SHH signaling pathway. To sum up, HT could inhibit apoptosis in I/R-induced AKI *via* activating SHH signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding Acknowledgement

Nantong University, Clinical Research Project (2019LY005).

References

1) Bellomo R, Kellum JA, Ronco C, Wald R, Martensson J, Maiden M, Bagshaw SM, Glassford NJ, Lankadeva Y, Vaara ST, Schneider A. Acute kidney injury in sepsis. Intensive Care Med 2017; 43: 816-828.
2) Ali T, Khan I, Simpson W, Prescott G, Townend J, Smith W, Macleod A. Incidence and outcomes in acute kidney injury: a comprehensive population-based study. J Am Soc Nephrol 2007; 18: 1292-1298.
3) Bihorac A, Brennan M, Ozrazgat-Baslanti T, Bozorgmehri S, Efron PA, Moore FA, Segal MS, Hobson CE. National surgical quality improvement program underestimates the risk associated with mild and moderate postoperative acute kidney injury. Crit Care Med 2013; 41: 2570-2583.
4) Uchino S, Kellum JA, Bellomo R, Doig GS, Morimatsu H, Morgera S, Schetz M, Tan I, Bouman C, Macedo E, Gibney N, Tolwani A, Ronco C. Acute renal failure in critically ill patients: a multinational, multicenter study. JAMA 2005; 294: 813-818.
5) Chang JW, Jeng MJ, Yang LY, Chen TJ, Chiang SC, Soong WJ, Wu KG, Lee YS, Wang HH, Yang CF, Tsai HL. The epidemiology and prognostic factors of mortality in critically ill children with acute kidney injury in Taiwan. Kidney Int 2015; 87: 632-639.
6) Loef BG, Epema AH, Smilde TD, Henning RH, Ebels T, Navis G, Stegeman CA. Immediate postoperative renal function deterioration in cardiac surgical patients predicts in-hospital mortality and long-term survival. J Am Soc Nephrol 2005; 16: 195-200.
7) Hobson CE, Yavas S, Segal MS, Schold JD, Tribble CG, Layon AJ, Bihorac A. Acute kidney injury is associated with increased long-term mortality after cardiothoracic surgery. Circulation 2009; 119: 2444-2453.
8) van Kuijk JP, Flu WJ, Chonchol M, Hoeks SE, Winkel TA, Verhagen HJ, Bax JJ, Poldermans D. Temporary perioperative decline of renal function is an independent predictor for chronic kidney disease. Clin J Am Soc Nephrol 2010; 5: 1198-1204.
9) Lassnigg A, Schmidlin D, Mouhieddine M, Bachmann LM, Druml W, Bauer P, Hiesmayr M. Minimal changes of serum creatinine predict prognosis in patients after cardiothoracic surgery: a prospective cohort study. J Am Soc Nephrol 2004; 15: 1597-1605.
10) Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. Lancet 2004; 364: 1814-1827.
11) Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest 2011; 121: 4210-4221.
12) Kosieradzki M, Rowinski W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplant Proc 2008; 40: 3279-3288.
13) Li X, Chen Z, Wu Y, Yan Y, Sun X, Yuan Q. Establishing an artificial pathway for efficient biosynthesis of hydroxytyrosol. ACS Synth Biol 2018; 7: 647-654.
14) SUN Y, ZHOU D, SHAHIDI F. Antioxidant properties of tyrosol and hydroxytyrosol saturated fatty acid esters. Food Chem 2018; 245: 1262-1268.
15) Gonzalez-Correa JA, Rodriguez-Perez MD, Marquez-Estrada L, Lopez-Villodres JA, Reyes JJ, Rodriguez-Gutierrez G, Fernandez-Bolanos J, De La Cruz JP. Neuroprotective effect of hydroxytyrosol in experimental diabetic retinopathy: relationship with cardiovascular biomarkers. J Agric Food Chem 2018; 66: 637-644.
16) Angeloni C, Malaguti M, Barbalace MC, Hrelia S. Bioactivity of olive oil phenols in neuroprotection. Int J Mol Sci 2017; 18: 2230.
17) Hashmi MA, Khan A, Hanif M, Farooq U, Perveen S. Traditional uses, phytochemistry, and pharmacology of Olea europaea (Olive). Evid Based Complement Alternat Med 2015; 2015: 541591.
18) Pan S, Liu L, Pan H, Ma Y, Wang D, Kang K, Wang J, Sun B, Sun X, Jiang H. Protective effects of hydroxytyrosol on liver ischemia/reperfusion injury in mice. Mol Nutr Food Res 2013; 57: 1218-1227.
19) Pei YH, Chen J, Xie L, Cai XM, Yang RH, Wang X, Gong JB. Hydroxytyrosol protects against Myo-

cardial ischemia/reperfusion injury through a PI3K/Akt-dependent mechanism. Mediators Inflamm 2016; 2016: 1232103.

20) Lafrance JP, Miller DR. Acute kidney injury associates with increased long-term mortality. J Am Soc Nephrol 2010; 21: 345-352.

21) Havasi A, Borkan SC. Apoptosis and acute kidney injury. Kidney Int 2011; 80: 29-40.

22) Sun HY, Wang NP, Halkos M, Kerendi F, Kin H, Guyton RA, Vinten-Johansen J, Zhao ZQ. Postconditioning attenuates cardiomyocyte apoptosis via inhibition of JNK and p38 mitogen-activated protein kinase signaling pathways. Apoptosis 2006; 11: 1583-1593.

23) Zheng X, Zhang X, Sun H, Feng B, Li M, Chen G, Vladau C, Chen D, Suzuki M, Min L, Liu W, Zhong R, Garcia B, Jevnikar A, Min WP. Protection of renal ischemia injury using combination gene silencing of complement 3 and caspase 3 genes. Transplantation 2006; 82: 1781-1786.

24) Zhu J, Yang Y, Wu J. Bcl-2 cleavages at two adjacent sites by different caspases promote cisplatin-induced apoptosis. Cell Res 2007; 17: 441-448.

25) Meng QH, Liu HB, Wang JB. Polydatin ameliorates renal ischemia/reperfusion injury by decreasing apoptosis and oxidative stress through activating sonic hedgehog signaling pathway. Food Chem Toxicol 2016; 96: 215-225.

26) Zhou D, Fu H, Liu S, Zhang L, Xiao L, Bastacky SI, Liu Y. Early activation of fibroblasts is required for kidney repair and regeneration after injury. FASEB J 2019; 33: 12576-12587.

27) Zhou D, Li Y, Zhou L, Tan RJ, Xiao L, Liang M, Hou FF, Liu Y. Sonic hedgehog is a novel tubule-derived growth factor for interstitial fibroblasts after kidney injury. J Am Soc Nephrol 2014; 25: 2187-2200.