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Original Article

Comparison of attenuating renal ischemia/reperfusion injury effects of raw and honey wine-processed Herba Siegesbeckiae

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Abstract: In this study, we aim at investigating the effects of raw Herba Siegesbeckiae (HS) and honey wine-processed HS (WHS) against oxidative injury and apoptosis in MaleWistar albino rats with renal ischemia/reperfusion injury (RIRI). Rats were randomized into groups as follows: (I) sham group (n = 10); (II) RIRI group (n = 10); (III) RIRI + silymarin (SI) (100 mg/kg) group (n = 10); (IV) RIRI + HS (400 mg/kg) group (n = 10); (V) RIRI + WHS (400 mg/kg) group (n = 10). For the RIRI + HS/WHS and RIRI + SI groups, rats were orally given with HS/WHS (400 mg/kg) and SI (100 mg/kg) once daily 10 days before induction of ischemia, followed by renal IRI. For the sham group and RIRI group, rats were orally given with equal volume of saline once daily 10 days before induction of ischemia, followed by renal IRI. Our results indicated that HS and WHS pretreatment remarkably decreased the blood urea nitrogen (BUN) and serum creatinine. WHS pretreatment also significantly suppressed the MDA release. On the other hand, HS and WHS effectively increase the activities of endogenous antioxidant enzymes. Western blot analysis showed that HS and WHS also reduced the p53 and caspase-3/-9 expression possibly through regulating the Wnt/β-cantenin. In addition, WHS showed better effect than HS against RIRI in rats, which can provide new sights for RIRI treatment.

Keywords: Herba Siegesbeckiae, ischemia/reperfusion, oxidative stress, apoptosis, Wnt/β-cantenin

Introduction

Herba Siegesbeckiae (HS) is a commonly used Chinese herbal medicine and the dried aerial parts of three species (*Siegesbeckiae orientalis* L., *Siegesbeckiae pubescens* Makino, *Siegesbeckiase glabrescens* Makino) are taken into use [1]. Siegesbeckiae are mainly used in the treatment of rheumatism, arthritis, detoxification, and it can also treat soreness and weakness of waist and knees, hemiplegia and other illnesses [2-4]. HS is usually processed with rice wine with the aim of decreasing the toxicity and enhanceing the efficacy. Previous reports investigated that HS shows multiple bioactivities including anti-bacterial, anti-oxidant and anti-inflammatory properties [5, 6].

Renal ischemia-reperfusion injury (RIRI) with high morbidity and mortality often occurs in clinical practice [7]. Improving the ability of organs to tolerate ischemic injury is of vital importance. RIRI is an inevitable pathological process which commonly occurs during kidney transplantation and causes acute kidney injury. The most important pathophysiology of such injury is the following inflammation and tubular epithelial cell apoptosis. The overproduction of reactive oxygen species (ROS), cell apoptosis, and the release of inflammatory cytokines always give rise to tissue damage [8]. A lot of strategies have been improved for RIRI treatment, but few are effective. Some antioxidants and anti-inflammatory Chinese herbs show favorable ability in alleviating renal injury. In normal kidney, the Wnt pathway is persistently active in cells of the papilla, where low oxygen tension causes cell stress and relatively high cell turnover [9]. However, in mouse models of RIRI, the Wnt pathway is widely activated, especially Wnt4 and Wnt7b [10].

In this study, a model of renal ischemic reperfusion in the rat was established. The effect of HS

and HS processed with honey and yellow rice wine on ischemic reperfusion induced renal damage and its renal-protective mechanism was investigated.

Materials and methods

Preparation of HS and WHS extracts

The raw herb HS (the dried aerial part of *S. orientalis*) was purchased from Shanghai ley's pharmaceutical co., LTD (Shanghai, China) and its authentication was confirmed by the School of Chinese Materia Medica, Beijing University of Chinese Medicine.

Preparation of WHS: 75 g of acacia honey (Baihua co., LTD, Beijing) and 75 g of yellow rice wine (Jinfeng co., LTD, Shanghai) mixed with ultrapure water were divided for 9 groups, one of which was mixed thoroughly HS (150 g) and then steamed it for 45 min. Eventually dry the herbs in the shade and repeat the above steps 9 times.

Preparation of HS and WHS extracts [11]: HS or WHS (100 g) was reflux-extracted twice with 50% ethanol (1:10, w/v) for 2 h each. The combined extracts were filtered after cooling and then concentrated under reduced pressure to remove the ethanol. The powdered HS (yield: 14.26%) and WHS (yield: 15.78%) extracts were obtained by lyophilizing of the concentrated samples with a Virtis Freeze Dryer (Huxi, Shanghai, China).

Animals

Male Wistar albino rats (200-250 g) purchased from Institute of Zoology (IOZ), Chinese Academy of Sciences (Beijing, China) were housed in an air-conditioned room with 12-h light and dark cycles, where the room temperature and relative humidity (65-70%) were kept constant. Rats were fed with standard laboratory chow with free access to water. All animal experiments were strictly performed according to the guide for the care and use of laboratory animals approved by the Bioethics Committee of the School of Chinese Materia Medica, Beijing University of Chinese Medicine.

Study design

Silymarin (SI, Sigma, Shanghai, China) was selected as the positive drug. Rats were randomized into groups as follows: (I) sham group (n = 10); (II) RIRI group (n = 10); (III) RIRI + SI (100 mg/kg) group (n = 10); (IV) RIRI + HS (400 mg/kg) group (n = 10); (V) RIRI + WHS (400 mg/kg) group (n = 10). For groups (IV, V), rats were orally given with HS and WHS (400 mg/kg body weight) once daily 10 days prior to the induction of ischemia, followed by renal IRI. Rats in group III were orally given with SI (100 mg/kg body weight) once daily 10 days prior to the induction of ischemia, followed by renal IRI. For the sham group and RIRI group, rats were orally given with equal volume of saline once daily 10 days prior to the induction of ischemia, followed by renal IRI.

Surgery and experimental design

Briefly, an upper abdominal midline incision was made and right nephrectomy was performed under anesthesia (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine; intraperitoneally, i.p.). The left renal pedicle was occluded for 45 min to induce ischemia and then subjected to reperfusion for 48 h. In the sham groups (n = 10), rats underwent right nephrectomy.

The animals were decapitated at either 48 h of the reperfusion period and trunk blood samples were collected for the analysis of blood urea nitrogen (BUN), and creatinine levels. Renal tissue samples obtained from each animal were stored at -80°C for the subsequent measurement.

Histopathological analysis of the renal

The renal was excised and then washed with saline. Renal samples were fixed in 10% neutral-buffered formalin solution for at least 24 h. The tissues were embedded in paraffin. The paraffin blocks were cut into 5-mm thick sections. The sections were stained with hematoxylin and eosin (H&E) for morphological evaluation under light microscope at 400× magnification.

Detection of BUN

BUN in serum was determined using a commercially available kit (Sigma, USA). The principle is based on the hydrolysis of urea in water by urease enzyme to form ammonia ions. The latter reacts in alkaline pH with sodium nitroprusside, hypochlorite and salicylate ions to yield a col-

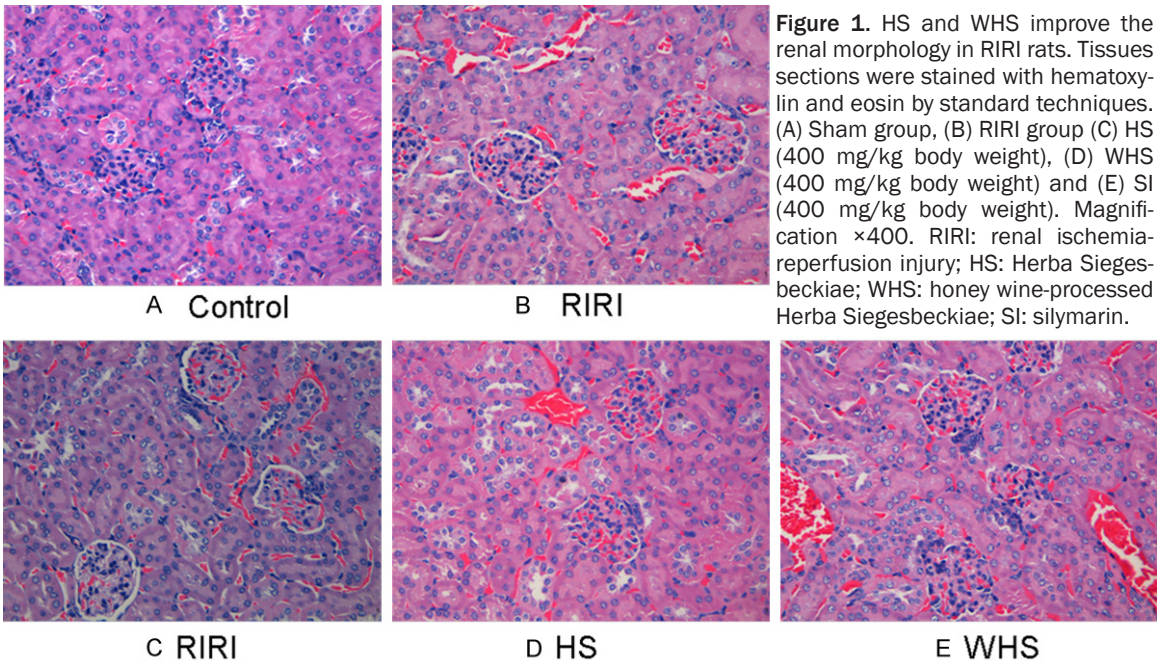

Figure 1. HS and WHS improve the renal morphology in RIRI rats. Tissues sections were stained with hematoxylin and eosin by standard techniques. (A) Sham group, (B) RIRI group (C) HS (400 mg/kg body weight), (D) WHS (400 mg/kg body weight) and (E) SI (400 mg/kg body weight). Magnification ×400. RIRI: renal ischemia-reperfusion injury; HS: Herba Siegesbeckiae; WHS: honey wine-processed Herba Siegesbeckiae; SI: silymarin.

ored indophenol complex with a proportional intensity to urea concentration in the specimen. The manufacturer's instructions were followed and the absorbance of the resultant green color was measured by a spectrophotometer set at 578 nm.

Detection of serum creatinine level

In the case of renal insufficiency, creatinine is retained in the blood and its excretion by kidneys is impaired. The assay is based on the reaction of creatinine with sodium picrate forming a red complex in a proportional fashion to creatinine concentration in the sample. Quantitative determination of serum creatinine was performed following the kit manual (Zelang Biotech Ltd., Shanghai, China). The absorbance was read at 510 nm using a spectrophotometer.

Biochemical analysis of the renal

Renal tissues were prepared in phosphate buffer (pH 7.4) to make 1:10 (w/v) homogenates. After centrifugation at 12000 g for 20 min at 4°C, the supernatants were collected for biochemical analysis. The levels of malondialdehyde (MDA), glutathione (GSH), catalase (CAT), glutathione peroxidase (GSH-Px) and SOD were determined by assay kits bought from Shanghai Zhongze Biotech Ltd. (China). The values were normalized by the protein concentration of the sample, which measured using bicinchoninic acid (BCA) assay.

Western blot

Renal homogenates (50 μg) were normalized by the Bradford method, resolved on 12% polyacrylamide gels, transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and probed with the appropriate primary and secondary antibodies. P53, caspase-3, caspase-9, Wnt4, Wnt7b, β-cantenin and GAPDH antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibody was a horseradish peroxidase-coupled anti-rabbit or anti-mouse IgG (Beverly, MA, USA). Membranes were visualized using an enhanced chemiluminescence western blotting detection kit (Zhongze Biotechnology Co., Ltd., Shanghai, China). The intensity of western blot bands was measured using the NIH Image J program.

Statistical analysis

The results were analyzed through the test ANOVA assumptions analyzed by one-way and followed by Turkey post test. In all of the cases a level of significance of 5% was used ($\alpha = 0.05$).

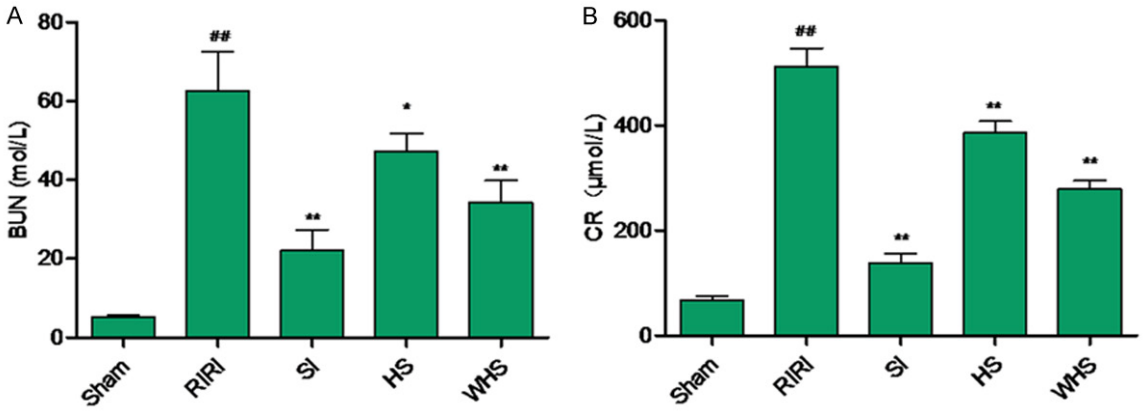

Figure 2. HS and WHS reduce serum BUN (A) and creatinine (B) levels in RIRI rats. Data were expressed as mean ± SD (n = 10). $^{\#\#}P < 0.01$, versus control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus RIRI group. RIRI: renal ischemia-reperfusion injury; HS: Herba Siegesbeckiae; WHS: honey wine-processed Herba Siegesbeckiae; SI: silymarin.

Results

HS and WHS improve renal morphology in RIRI rats

We firstly performed histological assessment of renoprotective effects of HS and WHS on RIRI rats by H&E staining. In **Figure 1A**, we can see the unbroken and normal renal tubules and glomeruli, and there is no tube type and swelling necrosis of epithelial cells in sham rats. In RIRI rats, renal tubular epithelial cell is swelling and bleeding, the vacuoles are degenerated in renal cortex. The loss of epithelial cells, a disorganization of cells and protein casting in dilated tubules can be seen in the lumen (**Figure 1B**). However, HS, WHS and SI can effectively improve renal morphology in RIRI rats. HS, WHS and SI improve the vacuolar degeneration and the necrosis in the most of the renal tubular epithelial cells to varying degrees (**Figure 1C-E**). Cell arrangements in HS, WHS and SI group are more consistent than that in RIRI rats. The structures of renal tubules and glomeruli are almost integrated and there are no tube type and swelling necrosis of epithelial cells in HS, WHS and SI pretreatment group.

Effect of HS and WHS pretreatment BUN and serum creatinine level

BUN and creatinine levels in serum are recognized as indexes of renal impairment, which are estimated using commercially available kit. As shown in **Figure 2A**, induction of renal I/R in rats notably promoted BUN and serum creatinine accumulation by 110.8%, and 73.3% compared with that of the control group ($P < 0.01$), respectively. Pretreatment of HS (400 mg/kg), WHS (400 mg/kg) and SI (100 mg/kg) can effectively decrease the BUN level in serum by 67.1%, 27.6% and 54.7% with statistical significance in comparison with that of RIRI rats ($P < 0.05$). In addition, administration of HS (400 mg/kg), WHS (400 mg/kg) and SI (100 mg/kg) prior to renal I/R also reduced the creatinine level by 73.8%, 21.4% and 49.2% (**Figure 2B**), as compared to the RIRI rats, respectively ($P < 0.01$).

HS and WHS relieve oxidative stress in RIRI rats

Lipid peroxidation and contents of endogenous antioxidants are common index of oxidative stress. It is shown in **Figure 3A** that a notable increase was observed in MDA concentration, which was significantly inhibited by WHS and SI treatment ($P < 0.01$). HS pretreatment also reduced the MDA level in RIRI rats but without statistical significance compared with the RIRI rats. We also estimated the GSH level of five groups by ELISA. GSH level in RIRI group was remarkably descended in comparison with that of control group. Administration of HS, WHS and SI notably increased the level of hepatic GSH compared to RIRI rats (**Figure 3B**).

In addition, we detected the activities of SOD, CAT, and GSH-Px in the renal of all experimental rats. SOD, CAT, and GSH-Px activities in RIRI rats were all decreased significantly compared with that of the sham rats (**Figure 3C-E**). WHS and SI could obviously increase SOD, CAT, and

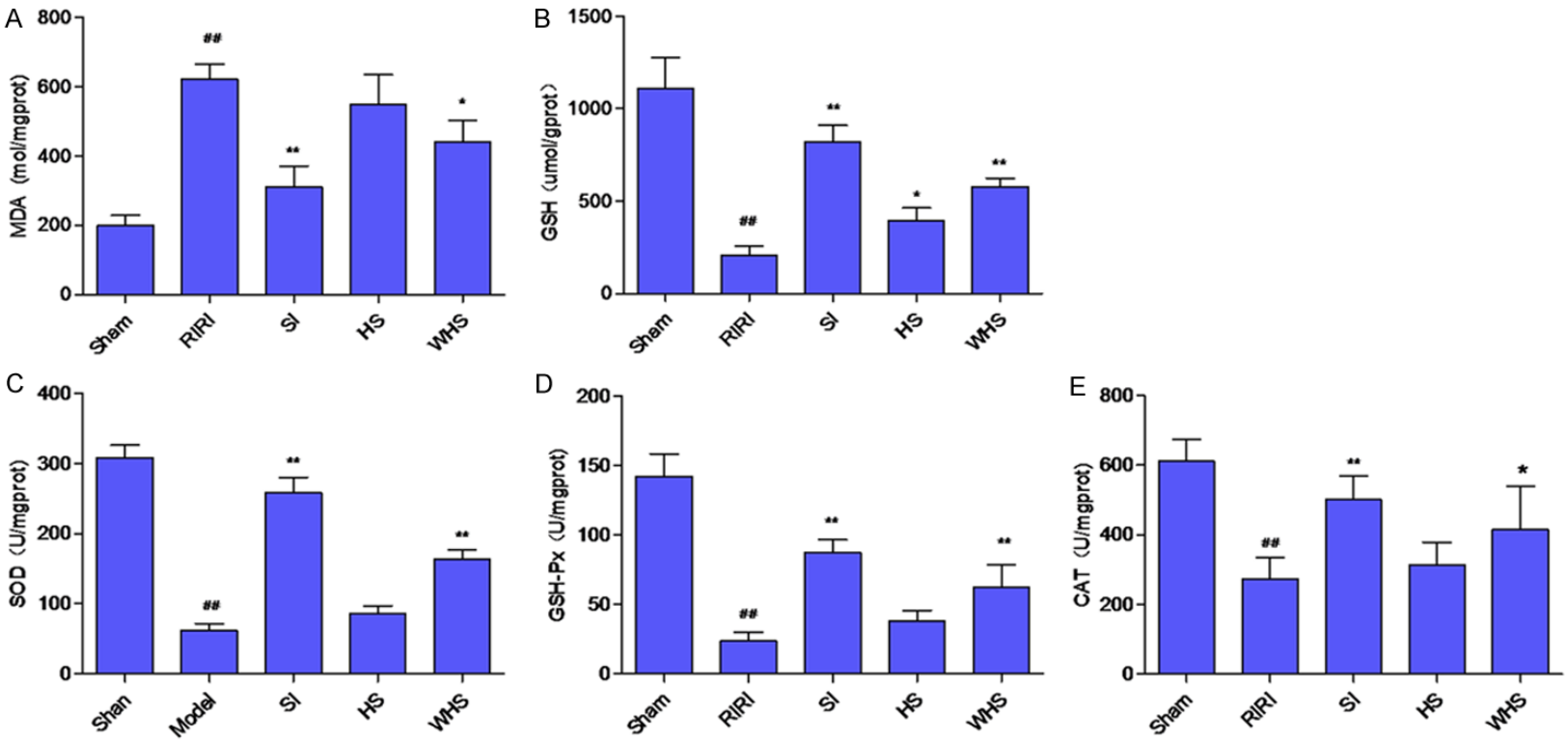

Figure 3. HS and WHS relieve oxidant stress in the renal of RIRI rats. Changes in renal (A) MDA content, (B) GSH content, (C) SOD activity, (D) GSH-Px activiy and (E) CAT activity. Data were expressed as mean ± SD (n = 10). $^{\#\#}P < 0.01$, versus control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus RIRI group. RIRI: renal ischemia-reperfusion injury; HS: Herba Siegesbeckiae; WHS: honey wine-processed Herba Siegesbeckiae; SI: silymarin. MDA: malondialdehyde; GSH: glutathione; SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase.

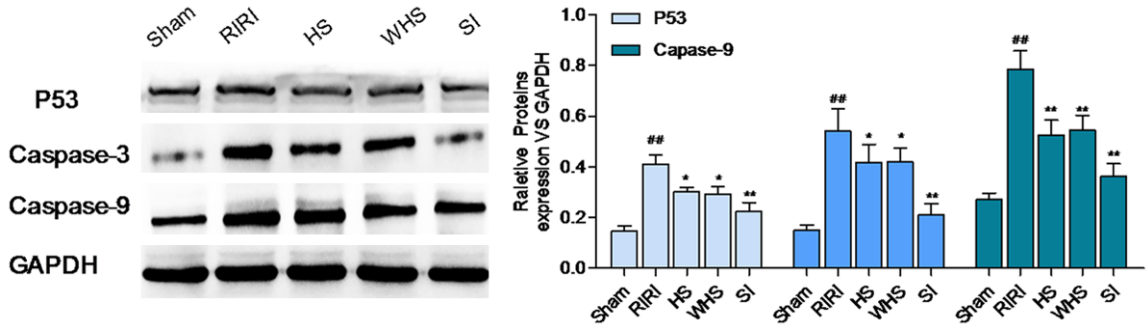

Figure 4. HS and WHS reduce protein expression of p53, caspase-3 and caspase-9 in the renal of RIRI rats. GAPDH was loading control. Data were expressed as mean ± SD (n = 10). $^{\#\#}P < 0.01$, versus control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus RIRI group. RIRI: renal ischemia-reperfusion injury; HS: Herba Siegesbeckiae; WHS: honey wine-processed Herba Siegesbeckiae; SI: silymarin.

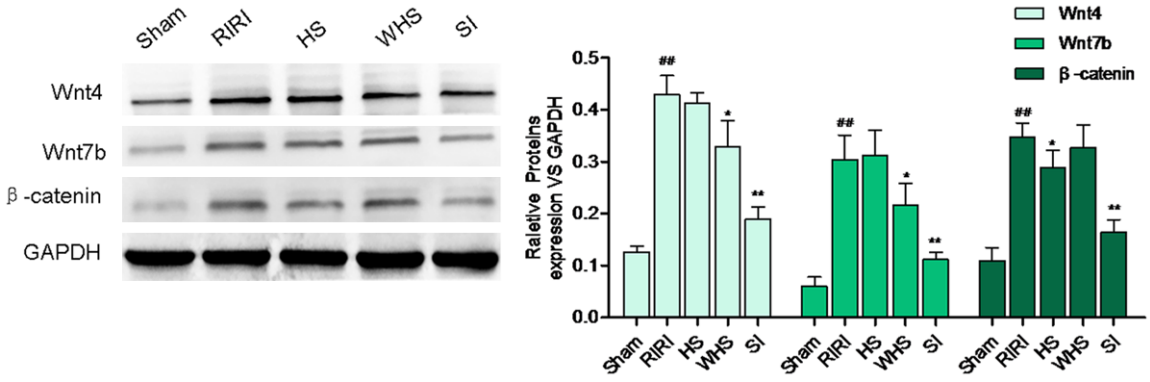

Figure 5. Effects of HS and WHS on Wnt/β-catenin signaling in RIRI rats. GAPDH was loading control. Data were expressed as mean ± SD (n = 10). $^{\#\#}P < 0.01$, versus control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus RIRI group. RIRI: renal ischemia-reperfusion injury; HS: Herba Siegesbeckiae; WHS: honey wine-processed Herba Siegesbeckiae; SI: silymarin.

GSH-Px enzyme activities in the renal of RIRI rats ($P < 0.01$), while HS showed no remarkable effect on SOD, CAT, and GSH-Px activities compared with the RIRI rats (**Figure 3C-E**). Taken together, these data suggested that HS and WHS could relieve the oxidative stress in the renal of RIRI rats to different degrees. Moreover, WHS pretreatment showed better efficacy in relieving the oxidative stress than that of HS.

HS and WHS reduce the levels of p53, caspase-3 and caspase-9 in the renal of RIRI rats

P53, caspase-3 and caspase-9 were correlated with cell apoptosis, we further determined the expressions of p53, caspase-3 and caspase-9 in the renal cells by western blot analysis. Compared with the sham rats, the protein level of p53, caspase-3 and caspase-9 were increased by 120.5%, 312.8% and 170.5% in the renal of RIRI rats, respectively (**Figure 4**). However, the protein levels of p53, caspase-3 and caspase-9 were significantly reduced in the HS, WHS and SI pretreatment groups ($P < 0.05$).

Effects of HS and WHS on Wnt/β-catenin signaling in RIRI rats

Wnt/β-catenin signaling is reported widely activated in acute kidney injury, we estimated the protein expression of Wnt4, Wnt7b and β-catenin in five groups by western blot analysis. As shown in **Figure 5**, remarkable increases of Wnt4, Wnt7b and β-catenin were observed in RIRI rats compared with the sham rats. WHS pretreatment can significantly decrease the expression of Wnt4 and Wnt7b expression compared with the RIRI rats ($P < 0.05$), while WHS showed no significant effect

on β-catenin expression However, HS only can dramaticlly reduce the β-catenin expression compared with the RIRI rats ($P < 0.05$).

Discussion

Raw Chinese medicinal herbs are usually performed to be processed before they are taken into clinical use or Chinese patent medicine. Multiple studies demonstrates that processing can enhance the efficacy and/or reduce the toxicity of raw herbs. In this study, the raw Herba Siegesbeckiae was processed with honey and yellow rice wine for 9 times and then proceeding for assessing renal-protective effects. Our previous study reported that Hexacosan-4-olide, 3,4-dihydroxyacetophenone, 3,4-dihydroxybenzaldehyde, p-hydroxyphenethyl alcohol, 5'-epimagnolin and senkyunolide H were obtained from WHS not in HS [12]. Firstly, we studied the histopathological analysis of the renal in sham, RIRI, HS, WHS and SI group. From the result (**Figure 1**), both HS and WHS can effectively improve the renal morphology in RIRI rats.

BUN and creatinine are two clinical examination indexes of renal function, which indicate the degree of renal injury. IR easily gives rise to the reduction of BUN and creatinine in a similar manner to previous studies [13, 14], while HS and WHS can significantly increase both of the BUN and creatinine levels. The kidney is extremely sensitive to changes in oxygen tension within its complex architecture, making it very prone to hypoxic injury when the renal artery is temporarily occluded [15]. MDA is one of the main lipid peroxidation products, and increased MDA level could reflect the degree of lipid peroxidation injury renal. Remarkable elevation in MDA level has been observed after RIRI treatment. Consistently, our results indicated that there was significant elevation in renal MDA accumulation in RIRI rats and pretreatment with WHS significantly reduced MDA level (**Figure 2**). GSH, GSH-Px, CAT and SOD are the most important endogenous antioxidant enzymes, and they are present in high concentrations in kidney cells [16]. GSH scavenges superoxide radicals and protects protein thiol (-SH) groups from oxidation [17]. Our results showed a significant decrease in the levels of SOD, GSH, CAT and GSH-Px in RIRI group. Pretreatment with WHS significantly increased the level of protective antioxidant enzymes and decreased lipid peroxidation, while HS only increased the level of GSH (**Figure 3**). This suggests that WHS exhibits better kidney-protective effect than HS against RIR-induced injury partly through its antioxidant role.

Caspase-3 and caspase-9 play an important part in the onset and evolution of cell apoptosis. Caspase-3 and caspase-9 can be activated by p53. It is reported that knockout of p53 effectively protect the kidney against the injury induced by cisplatin in rats [18]. Our study exhibited that after RIRI, considerable amounts of p53 and caspase-3/-9 were observed, WHS and HS pretreatment remarkably reduce the expression of p53 and caspase-3/-9 (**Figure 4**). The above results indicate that WHS and HS can inhibit apoptosis of renal cells through suppressing caspase-3/-9 expression. Wnt/β-catenin signaling pathway is a complex cell-to-cell communication pathway in multicellular organisms and tissues, which regulates cell proliferation, apoptosis and differentiation [9, 19]. Previous studies from independent groups indicated that Wnt4 expression was up-regulated in RIRI rats, implying that Wnt4 may be involved in the process of reparation [20]. Wnt ligands are regenerated after ischaemic kidney injury [21], particularly the Wnt7b. It is also reported that specific degradation of β-catenin in renal epithelium could result in exasperating RIRI. In our study, we found that WHS pretreatment caused an obvious decrease in Wnt4 and Wnt7a expression in RIRI rats, while HS only decrease the β-catenin expression (**Figure 5**).

In summary, our study shows that the administration of HS and WHS have a protective effect on renal I/R injury, which might be ascribed to the blockade of Wnt/β-catenin signal pathway, attenuating oxidative stress and increasing the anti-apoptosis capability. In addition, WHS exhibits better renoprotective effect than HS, which can provide new sights for renal IR injury.

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Disclosure of conflict of interest

None.

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