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Effect of Renshen polysaccharides on oxidative injury in kidney IR rabbits

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ABSTRACT

There is increasing evidence to suggest that reactive oxygen metabolites (ROMs) play a role in the pathogenesis of ischemia/reperfusion (I/R) injury in the kidney. This study was designed to determine the possible protective effect of Renshen polysaccharides (RSP) on renal ischemia/reperfusion (I/R) injury. Results showed that the polysaccharides of Renshen consisted mainly of glucose (29.21%), mannose (6.54%), rhamnose (4.34%), arabose (6.92%), galactose (18.41%). In addition, kidney lipid peroxidation level in IR rabbits were markedly increased, whereas antioxidant enzymes activities were significantly decreased. Renshen polysaccharides pre-treatment could decrease oxidative injury in kidney of IR rabbits.

1. Introduction

Ginseng (Renshen) (*Panax ginseng* C. A. Meyer) is one of the most valuable medicinal herbs in eastern Asian countries for the treatment of various ailments. Active compounds in ginseng include saponin (ginsenosides), acidic polysaccharides, peptides, polyacetylenes, alkaloids, and phenolic compounds (Attele, Wu, & Yuan, 1999). To date, numerous studies have demonstrated the pharmaceutical effects of *P. ginseng* on physical-, chemical-, and biological-stress (Shim et al., 2010), systemic immune function (Spelman et al., 2006), glucose metabolism (Lim et al., 2009), sexual function (Murphy & Lee, 2002), and mental capacity (Caso Marasco, Vargas Ruiz, Salas Villagomez, & Begona Infante, 1996). Several active compounds, including polyacetylenes, sesquiterpenes, peptidoglycans, polysaccharides, and approximately 40 ginsenosides, have been identified in *P. ginseng* (Lu, Yao, & Chen, 2009).

Despite significant advances in critical care medicine, acute renal failure remains a major clinical problem, causing considerable morbidity and mortality that has not decreased significantly over the last 50 years (Chatterjee & Thiemermann, 2003). Previous interventions against acute renal failure have proved to be largely negative, and dialysis remains the only effective therapy (Chatterjee & Thiemermann, 2003). Thus, the development of novel therapeutic interventions against acute renal failure has remained a topic of intense research interest (Chatterjee & Thiemermann, 2003; Venkataraman & Kellum, 2003). The mechanisms proposed to explain I/R injury include anoxia, release of reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (^-OH) during reperfusion, neutrophil accumulation, and subsequent release of additional ROS and lytic enzymes (Paller, Hoidal, & Ferris, 1984). ROS react with biomolecules such as cell membrane lipid as well as proteins, carbohydrates, nucleic acids, and thiols resulting in organic radical formation, lipid peroxidation, enzyme inactivation, glutathione oxidation, and cell destruction (Baud & Ardaillou, 1986; Zwemer et al., 2000). Cellular defense against oxidative injury is provided by several mechanisms. Antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), as well as nonenzymatic compounds such as reduced glutathione (GSH), ascorbic acid (AA), and α-tocopherol all help to cope with potential damage (Halliwell, Aeschbach, Loliger, & Aruoma, 1995). GSH is present in all mammalian cells, especially in renal cells, hepatocytes, and erythrocytes (Meister & Anderson, 1983). The increased production of ROS during I/R injury results in consumption and depletion of endogenous antioxidants. In this situation, the cells require exogenous antioxidant to protect them from ROS-induced damage.

In the present study, we investigate the effect of Renshen polysaccharides on oxidative injury in kidney IR rats.

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2. Material and method

2.1. Extraction of the crude polysaccharides hot water extraction (E1)

The Renshen powder was boiled in distilled water (1:5, w/v) at 100 °C for 2 h using a reflux condenser under reduced pressure. The hot extract was filtered with a nylon mesh bag (pore size 24 μm) and sequentially filtered with 0.45 μm Millipore filters. The filtrate was condensed using a rotary evaporator (BÜCHI Rotavapor R 200) to a minimal volume and then freeze-dried.

2.2. Analysis of monosaccharide composition by GC–MS

Polysaccharides (1 mg) was hydrolyzed with 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness. The dried carbohydrate samples were dissolved in 0.5 N NH_4OH (100 μL), held at room temperature for 10–15 min in reinforced 4 mL Pyrex tubes with Teflon lined screw caps. $NaBH_4$ (1 mg) was added, and the solution was maintained at 100 °C for 10 min, in order to reduce aldoses to alditols (Sassaki et al., 2008). The product was dried and excess $NaBH_4$ was neutralized by the addition of acetic acid or 1 M TFA (100 μL), which was removed following the addition of methanol (2×) under a N_2 stream in a fume hood. Acetylation of the Me-alditols was performed in pyridine–Ac_2O (200 μl; 1:1, v/v), heated for 30 min at 100 °C. The resulting alditol acetates were analyzed by GC–MS, and identified by their typical retention times and electron impact profiles. Gas liquid chromatography–mass spectrometry (GC–MS) was performed using a Varian (model 3300) gas chromatograph linked to a Finnigan Ion-Trap model 810 R-12 mass spectrometer, with He as carrier gas. A capillary column (30 m × 0.25 mm i.d.) of DB-225, held at 50 °C during injection and then programmed at 40 °C/min to 220 °C or 210 °C (constant temperature) was used for qualitative and quantitative analysis of alditol acetates and partially O-methylated alditol acetates, respectively (Sassaki, Gorin, Souza, Czelusniak, & Iacomini, 2005).

2.3. Animals and experimental design

45 rabbits were procured from local animal supplier and were kept in cages following animal keeping guidelines issued by the University. A normal 12L:12D light cycle was maintained. Temperature was maintained at 26 ± 3 °C throughout the experiment. Rabbits were acclimatized for one week initially and fed with normal diet and tap water ad libitum.

Animals were divided into the following groups: (1) sham group (SC); (2) I/R group; (3) I/R + RSP-pre-treated (600 mg/kg body weight) group. Each group consists of 15 animals. The experimental procedures were approved by the local legislation for ethics of experiments on animals. During ischemia and reperfusion, rectal temperature was maintained at about 37 °C. RSP extract (600 mg kg^{-1}; I/R + RSP group) or saline (I/R group) was pre-administered orally to rabbits for 30 days before ischemia reperfusion. On the 31st day, rats were fasted overnight. Under anesthesia, a midline laparotomy was made, and using minimal dissection, the left renal pedicle was isolated. The renal pedicle was occluded for 50 min to induce ischemia and then subjected for 8 h of reperfusion (I/R groups). In the sham-operated control group, renal pedicle was excised but left intact. In this group, the rats were treated with saline (sham control group).

Rabbits were sacrificed upon completion of ischemia/reperfusion; blood samples were collected and analyzed for blood serum biochemical indices. Kidneys were immediately removed and rapidly frozen in liquid nitrogen or fixed in paraformaldehyde for next studies.

2.4. Enzymes activities analysis

Serum tumor necrosis factor-α (TNF-α) were measured using double-ligand, enzyme-linked, immunosorbent assay (ELISA) kits.

Serum Scr and urea were estimated by using a full automatic biochemical analyzer.Glutathione (GSH) was estimated using the method of Sedlak and Lindsay (1968). The activity of superoxide dismutase (SOD) was assayed using the method of Marklund and Marklund (1974). The activity of glutathione peroxidase (GSH-Px) was assayed using the method of Lawrence and Burk (1976). Catalase (CAT) activity was measured following the method of Aebi (1984). Malondialdehyde (MDA) levels were determined in hepatic tissues by measuring the intensity of thiobarbituric acid reactive substance formation (Ohkawa, Ohish, & Yagi, 1979).

2.5. RT-PCR

Total RNA was collected by one-step method of Trizol to adjust RNA concentration of 200 μg/mL (Lu, Qin, Ye, & Yang, 2011). Those RNA were identified for their integrity by agarose gel electrophoresis. RT was carried out by reverse transcription kit of Omniscript™. According to SYBR from Green PCR kit instructions, 2.5 μl cDNA was used as templates for PCR. The amplification protocol comprised an initial period of denaturation at 95 °C for 10 min, 40 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 20 s, extension at 72 °C for 35 s, and a final period of extension at 72 °C for 10 min. RT-PCR amplification products were separated by a 1.5% agarose gel electrophoresis. The results were quantitatively analyzed by analytical system of Image Master Totallab V2.01 to calculate the relative content.

2.6. Western blot analysis

Twenty micrograms of protein was separated by 10% SDS-PAGE using a Bio-Rad mini-protein II electrophoresis, and transferred into polyvinylidene difluoride membrane (PVDF, Amersham Pharmacia Biotech, Piscataway, NJ). Blots were blocked (2 h) with 5% non-fat dry milk in Tris-buffered saline (TBS) at room temperature and then incubated overnight at 4 °C with antibody against HIF-1α protein (1:2000) (Sigma, St. Louis, MO, USA) diluted in blocking buffer. Blots were incubated with HRP-conjugated secondary anti-rabbit antiserum (Santa Cruz, CA, USA) diluted 1:5000 in TBS. After several washes with 0.1% TBS-Tween 20, immunoreactive proteins were visualized with an enhanced chemiluminescence (ECL) and captured on an X-ray film. Protein levels were quantitated using Biosense 300 software (Oberhaching, Germany). Each membrane was routinely stained with Ponceau S to ensure equivalent loading. Common sample were included on each blot to allow quantitative between-blot comparisons. Initial quantities were adjusted by subtracting the background, then normalized for any loading inequities based on the Ponceau S stained image of the gel, and finally adjusted for signal intensity differences among groups.

2.7. Histopathological analysis

Small pieces of kidney fixed in 10% buffered neutral formalin were processed for embedding in paraffin. Sections of 5–6 μm thickness were stained with hematoxylin and eosin, examined for histopathological changes (200×) under a compound microscope.

2.8. Statistical analysis

Data were analyzed using the SPSS package. Results are expressed as mean ± SEM with the experiment repeated at least

Table 1
Chemical composition of Renshen polysaccharides.

No.	Chemical composition	Percent
1	Glucose	29.21
2	Mannose	6.54
3	Rhamnose	4.34
4	Arabose	6.92
5	Galactose	18.41

Table 2
Effect of Renshen polysaccharides on serum MDA level in kidney IR rabbits.

Group	MDA (mmol/L)
Sham control (SC)	4.83 ± 0.33
IR	8.15 ± 0.72**
IR + RSP	5.62 ± 0.41##

** $p < 0.01$, group IR vs group SC.
$p < 0.01$, group IR + RSP vs group IR.

three times. Statistical evaluations were done using the analysis of variance (Anova). A p value of <0.05 was considered significant.

3. Result and discussion

The monosaccharide composition of Renshen polysaccharides was evaluated by GC–MS. According to Table 1, the polysaccharides of Renshen consisted mainly of glucose (29.21%), mannose (6.54%), rhamnose (4.34%), arabose (6.92%), galactose (18.41%).

One of the most important factors in pathophysiology of renal IR injury is reactive oxygen species (ROS), which especially increases in reperfusion phase (Li & Jackson, 2002; Paller et al., 1984; Akbaba et al., 2012; Sharifi et al., 2012). The endogenous antioxidants which are responsible for defense against ROS during reperfusion have an important role in decreasing IR injury (Li & Jackson, 2002). Superoxide dismutase (SOD) and catalase (CAT) are the most important antioxidant enzymes of tissues (Berg, Tymoczko, & Stryer, 2002). Glutathione (GSH), a free radical scavenger (Pincemail et al., 2000), plays a key role in maintenance of the cellular redox environment (Schafer & Buettner, 2001).

In this study, MDA level in kidney of RSP-pretreated and untreated IR rabbits were presented in Table 2. Compared with SC group, MDA level was significantly increased in the IR group, whereas an decrease was observed in MDA level in IR + RSP-pretreated group compared with untreated IR group.

GSH level in kidney of IR + RSP-pretreated and untreated rats were presented in Table 3. Compared with SC group, GSH level was significantly decreased in the IR group, whereas an increase was observed in GSH level in IR + RSP-pretreated group compared with untreated IR group.

The result of Renshen polysaccharides effects on serum SOD, CAT and GSH-Px in rabbits was presented in Table 4. In IR groups, the serum SOD, CAT and GSH-Px activities were significantly decreased compared to SC group. There was a significant increase in SOD, CAT and GSH-Px activities after 30 days of Renshen polysaccharides supplementation.

Traditional biomarkers of renal function, including blood urea nitrogen (BUN) and serum creatinine (SCr), rely primarily on the detection of impaired kidney function. Tumor necrosis factor (TNF, cachexin or cachectin formerly known as tumor necrosis factor-alpha or TNF-α) is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages, although it can be produced by other cell types as well. The primary role of TNF is in the regulation of immune cells. TNF, being an endogenous pyrogen, is able to induce fever, to induce apoptotic cell death, to induce sepsis (through IL1 and IL6 production), to induce cachexia, induce inflammation, and to inhibit tumorigenesis and viral replication. Dysregulation of TNF production has been implicated in a variety of human diseases, including Alzheimer's disease (Swardfager et al., 2010; Olorunnisola, Bradley, & Afolayan, 2012; Seidavi, 2011), cancer (Locksley, Killeen, & Lenardo, 2001), major depression (Dowlati et al., 2010), and inflammatory bowel disease (IBD) (Brynskov et al., 2002). Recombinant TNF-α is a bioactive protein intended for use in cell culture applications. TNF-α causes cytolysis and cytostasis of many tumor cell lines. TNF-α has a wide spectrum of activities, including chemotaxis of neutrophils, alteration of the endothelium, inhibition of anticoagulatory mechanisms, and promotion of angiogenesis.

Table 3
Effect of Renshen polysaccharides on serum GSH level in kidney IR rabbits.

Group	GSH
SC	142.8 ± 11.75
IR	67.92 ± 4.82**
IR + RSP	152.8 ± 13.29##

** $p < 0.01$, group IR vs group SC.
$p < 0.01$, group IR + RSP vs group IR.

Table 4
Effect of Renshen polysaccharides on serum SOD, CAT and GSH-Px level in kidney IR rabbits.

Group	SOD (U/mL)	CAT (U/mL)	GSH-Px (U/mL)
SC	269.4 ± 22.18	65.3 ± 4.82	68.84 ± 3.97
IR	137.1 ± 11.74**	30.16 ± 3.19**	35.17 ± 2.18**
IR + RSP	223.5 ± 20.68##	59.39 ± 3.22##	63.06 ± 3.63##

** $p < 0.01$, group IR vs group SC.
$p < 0.01$, group IR + RSP vs group IR.

The levels of serum urea, Scr and TNF-α were shown in Table 5. It could be found that the levels of serum urea, Scr and TNF-α were significantly increased in IR group animals compared to sham control group. Animals pre-administered with RSP at 600 mg/kg for 30 days displayed a remarked decrease in serum urea, Scr and TNF-α levels compared to the IR group ($p < 0.01$) (Table 5).

To adapt to hypoxia and to prevent ischemic injury, counter-regulatory mechanisms have evolved in the kidney, which are in part mediated by the two hypoxia-inducible transcription factors (HIF-1α and HIF-2α), which are stabilized under low oxygen tensions. HIF can initiate transcription of more than 100 genes, which are involved, for example, in glycolysis, angiogenesis, erythropoiesis, and cell cycle regulation or cell survival. Hypoxia is the most powerful stimulus for VEGF transcription. The hypoxia-dependent regulation of VEGF occurs at transcriptional level and is mainly mediated by the hypoxia-inducible transcription factor (HIF-1). In the kidney, HIF-1α has been localized to the tubular epithelia, whereas HIF-2α is predominantly located in endothelial, glomerular, and interstitial cells (Rosenberger et al., 2002). Concordantly, HIF-2α appears to be the HIF isoform controlling erythropoietin production in peritubular interstitial cells (Rankin et al., 2007; Warnecke et al., 2004; Al-Rejaie et al., 2012; Khan et al., 2011). In the presence of oxygen, two proline residues within the oxygen-dependent degradation domain of the a-chain of the

Table 5
Effect of Renshen polysaccharides on serum urea, Scr and TNF-α levels in kidney IR rabbits.

Group	Urea (mmol/L)	Scr (μmol/L)	TNF-α (ng/L)
SC	6.57 ± 0.59	31.52 ± 4.35	3.01 ± 0.29
IR	11.26 ± 1.36**	62.69 ± 5.87**	17.25 ± 1.37**
IR + RSP	8.73 ± 0.77##	47.46 ± 3.91##	6.84 ± 0.55##

** $p < 0.01$, group IR vs group SC.
$p < 0.01$, group IR + RSP vs group IR.

Table 6
Effect of Renshen polysaccharides on kidney HIF-1α mRNA and HIF-1α protein expression.

Group	HIF-1α mRNA	HIF-1α protein
SC	3.24 ± 0.28	0.21 ± 0.02
IR	8.13 ± 0.78**	0.19 ± 0.01
IR + RSP	25.48 ± 3.06##	0.14 ± 0.02#

** $p < 0.01$, group IR vs group SC.
# $p < 0.05$, group IR + RSP vs group IR.
$p < 0.01$, group IR + RSP vs group IR.

HIF protein are hydroxylated by at least three prolyl hydroxylase domain (PHD1–3) proteins. Hydroxylation of HIF leads to ubiquitination by the pVHL (von Hippel–Lindau protein) ubiquitin ligase, which targets HIF-α for proteasomal degradation. When molecular oxygen is not available for hydroxylation, HIF can accumulate in the cell and can promote transcription of its target genes (Kaelin & Ratcliffe, 2008).

The levels of HIF-1α mRNA expression in kidneys of sham control group animals were significantly higher than that of the sham control animals (Table 6). Renal HIF-1α protein expression level was decreased compared to sham control animals (Table 6). The levels of HIF-1α mRNA expression and HIF-1α protein expression in the kidneys of ANE-treated animals at 30 days were significantly higher or lower than that of the IR control animals.

Light microscopic evaluation of the sham group kidneys revealed a regular morphology of renal parenchyma with well designated glomeruli and tubuli. Histopathological examination revealed severe lesions in the kidney of untreated IR rabbits. These changes were characterized by dilated tubuli, loss of tubular lining epithelium and capillary congestion. RSP pre-treatment attenuated the development of all these lesions.

4. Conclusion

The present study clearly indicates that RSP pre-treatment attenuates the development of the ischemia/reperfusion-induced renal oxidative injury and suppresses elevated HIF-1α mRNA and HIF-1α protein expression in the kidney after the ischemia/reperfusion. It is reasonable to consider that the protective effect of RSP against the renal injury induced by ischemia/reperfusion is closely related to the inhibition of oxidative injury and HIF-1α mRNA and HIF-1α protein expression by this polysaccharides.

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