Hesperidin improves warm ischemia/reperfusion-induced oxidative renal injury in rats

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Received for publication April 07, 2008; accepted March 20, 2009

SUMMARY

Ischemia/reperfusion injury, which is commonly seen in the field of renal surgery or transplantation, is a major cause of acute renal failure. Previous studies showed that antioxidant treatments attenuated renal ischemia/reperfusion injury. The objective of this study was to examine the role of hesperidin in modulating reactive oxygen species induced inflammation and apoptosis after renal ischemia/reperfusion injury. Rats were subjected to right nephrectomy, 15 days later 45 min of renal ischemia and 24 h reperfusion with or without treatment with hesperidin. Renal function, inflammation and apoptosis were compared at 24 h after reperfusion injury. Hesperidin improved the renal dysfunction and reduced inflammation and apoptosis after ischemia/reperfusion injury. In conclusion, hesperidin shows potent anti-apoptotic and anti-inflammatory properties due to antioxidant property. These findings may have major implications in the treatment of human ischemic acute renal failure.

Key words: Antioxidant; Hesperidin; Ischemia reperfusion injury; Renal ischemia

INTRODUCTION

Postoperative acute renal failure in consequence of ischemia and reperfusion (I/R) injury can occur after kidney transplantation (Bouchier-Hayes et al., 1999). Ischemic cell injury in the kidney occurs during cardiovascular surgery, renal transplantation as well as the early allograft rejection subsequent to renal transplantation (Manuela, 2003). Excessive reactive oxygen species (ROS) generation occurs in I/R is proved in many biochemical and immunohistochernical studies. Generation of ROS, leading to dysfunction, injury, and renal cell necrosis (Chatterjee et al., 2000; Prabal and Chatterjee, 2007). Defense against free radical injury is provided by enzymatic (catalase, superoxide dismutase and glutathione peroxidase) and non-enzymatic (alpha tocopherol, vitamin C, allopurinol, dimethyl sulfoxide.) free radical scavengers (Mark et al., 1991; Devinder and Kanwaljit, 2004b). The protection provided by these free radical scavengers against ROS produced during injury further supports the hypothesis, ROS are involved in the cellular pathogenesis of I/R injury.

Thus, research efforts designed to prevent or ameliorate tissue injury have centered on inhibiting free radical generation during I/R injury.
Hesperidin is a major and active flavanone glycoside mainly isolated from citrus fruits (Cho, 2006). It is reported to possess anti-allergenic, radio protective and anti-oxidant activities (Naveen et al., 2005a; Hosseinimehr and Nemati, 2006). Moreover, hesperidin is shown to possess immunomodulator (Chia-Chou et al., 2007), and antihypertensive activities (Garg et al., 2007). When hesperidin is administered orally, it is hydrolyzed by intestinal microflora to yield a major active metabolite hesperitin (Cho, 2006).

So far, there are no findings to prove that treatment with hesperidin could improve the survival rate after renal warm I/R injury. In this study, we examined whether treatment with hesperidin improve the survival rate in a renal warm I/R injury using a rat model.

**MATERIALS AND METHODS**

**Animals**
Sprague-Dawley rats of either sex with body weight between 230 and 260 g were housed in an air-conditioned room with 12 h light and dark cycles, with free access to food and water ad libitum during the experiments. The institutional animal ethics committee approved the experimental protocol. All the experiments were conducted as per norms of CPCSEA.

**Grouping of animals**
The rats were divided into four groups each consisting of six animals.
Group 1: sham operated animals
Group 2: (I/R) on the day 1 animals subjected to right nephrectomy, later underwent 15 days vehicle treatment (0.5% sodium Carboxy Methyl Cellulose (CMC)), on the 16th day, animals subjected to 45 min of left renal ischemia followed by 24 h reperfusion.
Group 3: (I/R+HSP) on the day 1 animals subjected to right nephrectomy, later underwent 15 days Hesperidin treatment (100 mg/kg, p.o.) (Naveen et al., 2005b) on the 16th day, animals subjected to 45 min of left renal ischemia followed by 24 h reperfusion.
Group 4: (I/R + Vit.E) on the day 1 animals subjected to right nephrectomy, later underwent 15 days Vitamin E treatment (100 mg/kg/day, p.o) (Uma and Rao, 2005), on the 16th day, animals subjected to 45 min of left renal ischemia followed by 24 h reperfusion.

**Surgical procedure**
The progress of the experiment

<table>
<thead>
<tr>
<th>Day 1</th>
<th>15 days</th>
<th>Day 16</th>
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<td>Unilateral right nephrectomy</td>
<td>Drug treatment</td>
<td>45 min ischemia (left kidney) + 24 h reperfusion</td>
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After ketamine (100 mg/kg, i.p.) as general anesthesia to animals, which were starved for 12 h prior to surgical procedure, right nephrectomy was performed through a right flank incision (2 cm) 15 days before the ischemic procedures in the contralateral kidneys. In the sham-operated rats, right nephrectomy and left laparotomy were performed without making the left kidney ischemic.

Renal ischemia required performing a left flank incision and dissecting the left renal pedicle to expose the renal vessels. Nontraumatic vascular clamps were used to stop blood flow (artery and vein) during 45 min. Reperfusion was established by removing the clamp.

The abdominal wall (muscular layer and skin) was closed with 4.0 mononylon suture.

At the end of reperfusion period, blood samples were collected and used for the measurement of renal function and TNF-α. The abdomen was opened and the kidneys were collected for further analysis.

**Measurement of blood pressure**
During treatment schedule Systolic blood pressure
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(SBP) was measured from tail vein by tail cuff instrument (latica) on the day 1 (on the day of unilateral nephrectomy), 7, 15 (before ischemia) and 17 (after 24 h of reperfusion).

Renal function
After treatment schedule, blood was collected from all the animals. Serum samples were assayed for blood urea nitrogen (BUN) (DAM method) and serum creatinine (Jaffe’s method) by using standard diagnostic kits (Span Diagnostics, Gujarat, India).

TNF-α quantitation by ELISA
Levels of TNF-α in serum were determined using an enzyme-linked immunosorbent assay (ELISA) (Endogen, Mouse TNF-α kit, USA) according to the manufacturer’s instructions.

MPO activity
MPO (Myeloperoxidase) activity was measured in tissues in a procedure similar to that documented by Hillegas et al. (1990). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM Phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles with sonication between cycles, the samples were centrifuged at 41,000 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM Phosphate buffer, o-dianisidine, and 20 mM H2O2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as mOD/min.

Biomarkers of oxidative stress
GSH was estimated by the method of Moran et al. (1979). MDA was estimated by the method of Slater and Sawyer (1971). SOD was estimated by the method of Misra and Fridovich (1972).

Tissue NO levels
The level of nitric oxide (NO) was estimated by the method of Lepoivre et al. (1990). To 0.5 ml of tissue homogenate, 0.1 ml of sulphasalicylic acid was added and vortexed well for 30 min. The samples were then centrifuged at 5,000 rpm for 15 min. The protein-free supernatant was used for the estimation of nitrite levels. To 200 ml of the supernatant, 30 μl of 10% NaOH was added, followed by 300 ml of Tris-HCl buffer and mixed well. To this, 530 ml of Griess reagent was added and incubated in the dark for 10 - 15 min and the absorbance was read at 540 nm against a Griess reagent blank. Sodium nitrite solution was used as the standard. The amount of nitrite present in the samples was estimated from the standard curves obtained.

Histopathology
For light microscopic evaluation, kidneys were fixed in 10% phosphate buffered formalin. Paraffinembedded specimens were cut into 6 mm thick sections and stained with hematoxylin & eosin (H & E). The kidneys were examined under a light microscope (Olympus Bioxl) for the presence of tubular changes and interstitial inflammatory cell infiltration, by an observer blinded to the animal treatment group.

DNA fragmentation
Genomic DNA was extracted from renal cortices using DNA extraction kit (DNeasy kit, Axygen). Ten micrograms of DNA were electrophoresed on a 2% agarose gel. Fragmented DNA was visualized by ethidium bromide under an UV light source.

Statistics
All the data are expressed as mean ± SEM. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student’s t-test as appropriate using computer based fitting program (prism, Graphpad).
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Measurement of blood pressure
Fig. 1 shows Systolic Blood Pressure (SBP) of different groups of animals was measured on the day 1, 7, 15 (before ischemia) and 24 h after reperfusion. On the day of unilateral nephrectomy (day 1st), the sham operated animals showed mean SBP values of 121.3 ± 1.64 mmHg, which did not significantly changed after reperfusion. Animals underwent I/R showed significant rise ($P < 0.001$, $n = 6$) in mean SBP (151.2 ± 2.81 mmHg) after ischemia reperfusion in comparison to their mean SBP on the day 1. However, hesperidin treatment had significantly ($P < 0.001$) prevented rise in the mean SBP in comparison I/R group. Treatment with Vit.E in rats subjected to renal warm I/R prevented the rise in SBP, but not significant as hesperidin.

Renal function
To determine the beneficial effects of hesperidin on renal function, we examined serum creatinine and BUN levels in various groups of animals. The serum creatinine and BUN were significantly increased by 45.29% and 20% respectively in animals that underwent I/R animals as compared to sham operated group (Figs. 2A and B), indicating a significant degree of glomerular dysfunction mediated by renal I/R. Treatment with hesperidin produced a significant reduction in serum creatinine ($P < 0.01$) and BUN ($P < 0.01$) in comparison to I/R group.

RESULTS

Measurement of blood pressure

Fig. 1. Effect of hesperidin on systolic blood pressure in rats subjected to 45 min of ischemia in renal artery and vein followed by 24 h reperfusion. Unit: mmHg. Values are expressed as mean ± S.E.M. for 6 animals in the group. Comparisons are made between: sham and I/R, I/R + HSP, I/R + Vit. E. The symbols represent statistical significance:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Fig. 2. (A) Effect of hesperidin on serum creatinine in rats subjected to 45 min of ischemia in renal artery and vein followed by 24 h reperfusion. Unit: mg/dl. Values are expressed as mean ± S.E.M. for 6 animals in the group. Comparisons are made between: sham and I/R, I/R + HSP, I/R + Vit. E. The symbols represent statistical significance:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (B) Effect of hesperidin on BUN levels in rats subjected to 45 min of ischemia in renal artery and vein followed by 24 h reperfusion. Unit: mg/dl. Values are expressed as mean ± S.E.M. for 6 animals in the group. Comparisons are made between: sham and I/R, I/R + HSP, I/R + Vit. E. The symbols represent statistical significance:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Differences were considered to be statistically significant when $P < 0.05$. 

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Treatment with Vit. E in rats subjected to renal warm I/R improved renal function, but not significant as hesperidin.

**Markers of inflammation**
To determine the beneficial effects of hesperidin in prevention of inflammatory reactions, we examined serum level of TNF-α and MPO activity in kidney tissues. The serum TNF-α level was significantly ($P < 0.001, n = 6$) increased by 3 folds ($171.00 \pm 11.08$ pg/ml) in animals that underwent I/R animals as compared to sham operated group ($64.67 \pm 5.38$ pg/ml) (Fig. 3). Also, MPO activity was significantly ($P < 0.001, n = 6$) increased (4.63 fold) in animals that underwent ischemia compared to sham-operated animals (Table 1). Treatment with hesperidin significantly reduces serum TNF-α level ($P < 0.01$, $n = 6$) and MPO activity ($P < 0.05, n = 6$) in comparison to I/R group. Treatment with Vit. E in rats subjected to renal warm I/R reduced the tissue MPO activity and serum level of TNF-α, but not significant as hesperidin.

**Biomarkers of oxidative**
Renal I/R produced a significant increase in MDA levels ($147.80 \pm 4.71$ nM/mg of tissue), as compared to sham operated animals ($87.00 \pm 4.05$ nM/mg of tissue). Treatment with hesperidin produced a significant reduction in MDA level ($111.50 \pm 6.83$ nM/mg of tissue, $P < 0.001, n = 6$) in renal I/R + HSP group animals in comparison to I/R animals. Renal I/R significantly ($P < 0.001, n = 6$) decreased the antioxidant enzymatic activity of GSH ($278.30 \pm 4.24 \mu$g/g of tissue), CAT ($1141 \pm 21.08$ nM of H$_2$O$_2$ consumed/mg of tissue) and SOD ($56.50 \pm 2.59$ U/mg of tissue). This reduction was significantly improved by treatment with hesperidin ($345.20 \pm 11.87 \mu$g/g of tissue of GSH, $1283 \pm 42.98$ nM of H$_2$O$_2$ consumed/mg of tissue) and SOD ($56.50 \pm 2.59$ U/mg of tissue of CAT, and $86.53 \pm 3.0$ U/mg of tissue of SOD, respectively) in comparison to I/R group (Fig. 4(A) and 4(B)). Treatment with Vit. E in rats subjected to renal warm I/R improved tissue levels of biomarkers of oxidative stress, but not significant as hesperidin.

**Tissue NO levels**
Renal I/R resulted in a significant decrease in the tissue levels of nitrite ($123.8 \pm 9.56$ nM/mg tissue, $P < 0.05, n = 6$) in comparison with values obtained from the tissue of sham-operated animals ($156.7 \pm 5.87$) (Table 1). However, decreased nitrite levels mediated by renal I/R were increased significantly ($174.1 \pm 5.08$, $P < 0.001, n = 6$) after administration of hesperidin in comparison to I/R animals (Table 1). Treatment with Vit. E in rats subjected to renal...
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Warm I/R improved tissue NO level, but not significant as hesperidin.

Histopathology
Light microscopic evaluation of the sham-operated groups revealed a regular morphology of renal parenchyma with well-designated glomeruli and tubuli (Fig. 5A). In I/R group, the interstitial hemorrhage, dilated tubuli and prominent glomerular degeneration followed by atrophy revealed that I/R caused a severe glomerular, tubular and interstitial damage. Tubular dilation was present throughout the tissue (Fig. 5B). In the hesperidin-treated I/R group, there was a significant regeneration in all
features of the injury. Reduced tubular dilation, loss of interstitial hemorrhage and glomerular atrophy were the regenerated features (Fig. 5C).

DNA fragmentation
Necrosis was evaluated by DNA fragmentation analysis. The typical DNA laddering activity was observed in I/R group, which indicates cell necrosis. Treatment with hesperidin decreased I/R-induced DNA fragmentation. Treatment with Vit. E in rats subjected to renal warm I/R was failed to protect tissue necrosis as compared to hesperidin (Fig. 6).

DISCUSSION

In renal transplantation, the problem is the onset of I/R, when the transplantation requires a long interval as a consequence of using a brain dead donor’s kidney. The production of ROS during I/R of the kidney is one of the major causes contributing to acute renal failure (Devinder et al., 2004; Abdurrahman et al., 2006). Acute renal failure produced by I/R is characterized by major declines in glomerular filtration rate, accumulation of toxic metabolites, disturbance of electrolyte homeostasis, extensive tubular damage, inflammatory cell infiltration and tubular cell necrosis (Devinder and Kanwaljit, 2004; Abdurrahman et al., 2006b). Moreover, free radicals cause DNA scission and

![Fig. 5. Microscopic observations of kidneys tissue sections with BIOXL light microscope showing morphological changes. Images were taken under light microscopy using hematoxylin and eosin (×10). (A) sham-operated (B) I/R (C) I/R + HSP.](image)

![Fig. 6. DNA fragmentation analysis revealed typical laddering of fragmentated DNA in I/R group. Hesperidin treatment decreased the laddering pattern.](image)
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base modification, lipid peroxidation, protein damage and inactivation by their chemical modification (Devinder and Kanwaljit, 2004b; Sadk et al., 2005).

In this in vivo study, renal I/R caused significant increase in the renal MDA levels as an indicator of lipid peroxidation and depleted the anti-oxidant enzyme such as reduced glutathione, catalase and superoxide dismutase. Increase in lipid peroxidation can lead to nerve and smooth muscle membrane damage (Seiji et al., 2005). These alterations can increase tubular permeability with loss of membrane exchange functions with consequent impairment of the renal function (Ernani et al., 2002). Similar findings have also been seen in our study. Impaired renal function leading to significant increase in serum creatinine and BUN levels in I/R group compared to sham operated group. Previous experimental studies demonstrated that, antioxidant treatment can improve antioxidant enzyme pool, reduces ROS and protect against reperfusion injury (Devinder and Kanwaljit, 2004a; Kent et al., 2004; Seiji, 2005; Matsuyama, 2006; Zehra et al., 2007). Studies also proved that, the phenolic -OH group of bioflavonoids reacts with lipid peroxide radicals which terminate the lipid peroxidation chain reaction (Husain et al., 1987). Our study also shows hesperidin treatment significantly improved antioxidant enzyme pool, improved renal function and decreased MDA levels in treated animals compared to I/R group alone. Suggesting anti-oxidant properties of hesperidin, which may plays a significant role in protecting the renal microvasculature after I/R.

In our study, there was a significant rise in systolic blood pressure (SBP) during reperfusion period in I/R rats in comparison to sham operated animals. Studies proved that ROS are also implicated as the cause of damage to endothelial cells and hypertension (Esra et al., 2006). Endothelial cells produce less bioactive NO in presence of higher oxidative stress. Studies also proved that gene like, NOX-2 reduces endothelium derived relaxations and increases ROS generation simultaneously, in blood vessels of mice (Christopher, 2002). Ang II is a known stimulant for generation of ROS (Lilach et al., 2001). Moreover, ROS stimulates renin release from the juxtaglomerular apparatus by means of oxidized low-density lipoproteins and lipoproteins (Jan et al., 1997; Christopher, 2002). This provides a potential positive feedback loop whereby Ang II could stimulate oxidative stress that would release renin to generate more Ang II. Collectively leads to progressive rise in blood pressure and vasoconstriction. Studies show that, increase in hypertension severely changes morphology of kidney (Jean-Jacques et al., 2003). It is also confirmed by our histopathological results. However, administration of anti-oxidant like melatonin reduces hypertension in renal I/R rats (Esra et al., 2006). In this study, hisperidin treatment significantly prevented rise in hypertension during reperfusion period in treated animals compared to I/R animals.

A large number of work in animal models as well as some pathologic analysis of human biopsies demonstrate that ischemia is marked by a robust inflammatory response in tissues and contributes to the resultant tissue injury (Joshua, 2007). The renal tubular epithelium also generate mediators like IL-6, IL-1, TGF-β, cytokines and chemokines that potentiate inflammation following ischemic injury (Joseph and Anna, 2008). We measured the inflammatory response as tissue MPO activity and serum concentration of TNF-α. There was a significant increase in MPO activity of kidney tissue and serum TNF-α level in I/R group in comparison to sham-operated group. Increased MPO activity further produces ROS and hypochlorous acid, which exert a strong destructive effect on kidney tissues. Inflammatory agent such as TNF-α induces activation of NF-κB. Deregulation of NF-κB and its dependent genes has been associated with toxic shock, graft rejection and cancer (Ahmet et al., 2004). Moreover, TNF-α induces secretion of Monocyte chemoattractant protein (MCP)-1. MCP-1chemotactically recruits monocytes to sites of inflammation, which may further enhance MPO
activities (Jian et al., 2008). However, Perianayagam et al., demonstrated that antioxidants like melatonin reduce TNF-α production (Zehra et al., 2007).

In this study also, we observed that increased markers of inflammation (MPO activity and serum TNF-α) were significantly reduced by hesperidin treatment. Thus, our data indicates that hesperidin reduce inflammatory responses after renal I/R.

Inflammatory reactions increase the activity of iNOS mRNA in epithelial tubular cells. Studies showed that elevated expression of iNOS is accompanied by reduction in the number of cells that express eNOS (Manuela, 2003; Yagmurdur et al., 2008). NO is generally beneficial, but in presence of oxidative stress, it is potentially toxic. Under oxidative stress conditions, NO reacts with superoxide to produce peroxynitrite (Walker et al., 2000). In our study there was a significant decrease in the levels of NO in kidney tissues of rats underwent I/R compared to sham-operated group. If the ratio ROS/NO increases, which further activates tissue phosopholipase A₂ and thereby synthesizing inflammatory mediators (Ernani et al., 2002). In response to ROS the outer membrane of mitochondria becomes permeabilized, resulting in the translocation of Bax from cytosol to the mitochondria and the release of cytochrome c occurs. Release of cytochrome c into the cytosol leads to form the apoptosome which stimulates the activation of procaspase-9 and procaspase-3. Active caspase-3 activates the caspase activated DNAase, leading to DNA fragmentation (Manuela, 2003; Hui et al., 2008). Studies also proved that, high-level ROS cause necrotic cell death, in which the cellular contents are released into the surrounding environment, and some of the products released can induce inflammation, which is consistent with our results (Makiya, 2008). In our study, high degree of cell necrosis was observed in DNA samples of I/R group as compared with sham-operated group. However, animals treated with hesperidin showed high levels of NO after I/R. But, significant reduction in levels of inflammatory markers (MPO and TNF-α) and inhibition of cell necrosis (DNA fragmentation) caused by I/R was observed in animals treated with hesperidin in comparison to I/R group, shows that hesperidin inhibits the formation of peroxinitrite by inhibiting ROS.

In conclusion, ROS levels increase while SOD, catalase, and GSH levels decreases in renal ischemia reperfusion. Because of ROS peroxinitrite, blood pressure, inflammatory reactions and cell necrosis is also increases in renal ischemia reperfusion. Hesperidin lessens oxidative stress by increasing the levels of SOD, catalase and GSH. Besides, the use of hesperidin as an antioxidant drug can protect kidneys against renal ischemia reperfusion injury, which is an important issue in renal transplantation. However, it is also possible to speculate on many other mechanisms, such as changes in the level of other substances that attenuate oxidative stress but are to be detected.

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