Evaluation of Vitamin C Supplementation on Kidney Function and Vascular Reactivity Following Renal Ischemic Injury in Mice

Yao-Bin Zhu\textsuperscript{a} Ya-Ping Zhang\textsuperscript{a} Jing Zhang\textsuperscript{a} Yan-Bo Zhang\textsuperscript{b}

\textsuperscript{a}Pediatric Heart Center, Beijing Anzhen Hospital, Capital Medical University, Beijing; \textsuperscript{b}National Clinical Research Center of Cardiovascular Diseases, State Key Laboratory of Cardiovascular Disease, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

Key Words
Ischemia/reperfusion injury • Vascular reactivity • Vitamin C • Oxidative stress

Abstract

\textbf{Background/Aims:} Renal ischemia/reperfusion injury (IRI) is a very common clinical event and usually leads to ischemic acute renal failure (ARF). In the present study, we investigated the protective role of vitamin C in renal function and renal arterial relaxation following ischemic injury. \textbf{Methods:} IRI model in mice was used. Various biochemical parameters including nitric oxide (NO), reduced glutathione (GSH), total reactive oxygen species (ROS) level and superoxide dismutase (SOD) were examined. Doppler was used to investigate renal arterial resistance. The isolated renal arterial rings served for hypoxia/reoxygenation analysis. Acetylcholine (ACh) and sodium nitroprusside (SNP)-induced relaxations of isolated renal arterial rings were exerted. \textbf{Results:} Vitamin C supplementation preserved kidney morphology and renal function following IRI. It was shown that pretreatment with vitamin C for mice subjected to IRI significantly elevated renal NO and GSH levels after reperfusion. Meanwhile, vitamin C administration decreased resistance index of renal artery and ameliorated oxidative stress secondary to IRI. The total ROS level in renal artery was decreased whereas the renal arterial SOD expression was increased by vitamin C supplementation following IRI. Pretreatment with vitamin C significantly potentiated the ACh and SNP-induced relaxations in both control and hypoxic renal arterial rings. \textbf{Conclusion:} Vitamin C protects kidney function and renal arterial reactivity against IRI. The protective role of vitamin C is linked to ROS, SOD, GSH and NO levels in renal ischemic injury.
**Introduction**

Acute renal failure (ARF) is a very common event which usually leads to development of chronic kidney disease [1, 2]. Ischemia/reperfusion injury (IRI) is an unavoidable consequence of organ transplantation procedure [3]. The inflammatory response involved in IRI could expose the organ to immune responses and cause delayed graft function and allograft rejection [4]. There is convincing evidence revealing the role of oxidative stress which activates different signaling pathways in renal ischemic injury [5, 6]. Oxidative stress represents the increased presence of various free radicals that could not be buffered by the antioxidant capacity consisting of enzymatic and non-enzymatic components. Renal tissue injury during ischemia/reperfusion comes as a result of oxidative damage of proteins and DNA [7]. Meanwhile, enhanced vasoconstriction has been documented as a result of increased reactive oxygen species (ROS) and ROS could also impair vascular reactivity due to declining nitric oxide (NO) bioavailability [8]. It is well-known that NO participates in the pathophysiology of ARF and plays a great role in renal IRI [9-15]. The cross talk between ROS and NO has been well elucidated [16, 17]. The reaction between O$_2^-$ and NO leads to inactivation of NO as well as the generation of peroxynitrite species [18]. Reduced glutathione (GSH), which is an important antioxidant, protects tissues and cells against oxidative stress. GSH is present at high concentration in all mammalian tissues, especially in renal cells [19]. Superoxide dismutase (SOD) is the main endogenous antioxidant enzyme which converts superoxide anion to hydrogen peroxide [19]. It has been reported that GSH level and SOD activity are diminished in IRI and hypoxia-reoxygenation process [20, 21].

Since renal IRI is a challenging problem, deciphering the protective strategy that regulates oxidative stress and related molecular signals has attracted tremendous interest. It is evident that augmentation of the antioxidant defense mechanism has a protective role in renal ischemic tissue [22-25]. Vitamin C, a natural water-soluble antioxidant, has the potential to intervene in the development of cardiovascular disease by modulating redox steps [26]. Vitamin C could react rapidly with O$_2^-$ to play an antioxidant role in cytosol and extracellular matrix. It has been reported that vitamin C also scavenges peroxynitrite very effectively to prevent the formations of nitrotyrosine, nitrotryphophan and nitrated lipids [27]. In addition, intra-arterial administration of high dose of vitamin C results in improved vasodilation in hypertensive patients [28]. In light of the evidence, this study was designed to evaluate the effect of vitamin C on renal function and renal arterial relaxation following ischemic injury.

**Materials and Methods**

All procedures were carried out according to a protocol approved by the animal care and use committee of Capital Medical University. Animals were maintained and received care in Laboratory Animal Care Center of Anzhen Hospital, Capital Medical University.

**Ischemic/reperfusion injury model**

C57/BL background mice were purchased from Ke-Xing Animal Center (Beijing, China). Animals were housed in standard animal care rooms with 12-hour light-dark cycles and were allowed free access to food and water. In this study, 8-10 weeks old mice were used.

A midline incision in the abdomen was made and both renal pedicles were dissected to expose the renal vessels. Renal IRI was induced by clamping both renal arteries with non-traumatic vascular clamps for 45 min. Reperfusion was established by removing the clamps and then the abdominal incision was closed. Mice were killed by rapid cervical dislocation 48 h after reperfusion.

**Experimental design**

In vivo animal experiment, the mice were divided into four groups: 1) control group (Con); 2) IRI
group; 3) control group in presence of vitamin C (Con + VC); 4) IRI in presence of vitamin C (IRI + VC). Vitamin C (57 mg/kg/day) was added into drinking water and the dose of vitamin C was chosen on the basis of a preliminary study [29]. In vitro renal arterial reactivity experiment, there were also four groups: 1) control group (Con); 2) hypoxia/reoxygenation group (Hypo); 3) control group in presence of vitamin C (Con + VC); 4) hypoxia/reoxygenation in presence of vitamin C (Hypo + VC).

**Doppler detection**

Doppler was performed using a Vevo 2100 Imaging System (Visual Sonics, Canada) as previously described [30]. Mice were immobilized on a heating platform to maintain body temperature at 37°C and continuously anesthetized by isoflurane inhalant. Heart rate and respiratory rate were continuously monitored by electrocardiogram (ECG) electrode. Peak systolic and end-diastolic renal arterial blood velocities were measured using Color and PW Doppler-mode at angle of 60 degree in supine position. Renal resistance index was automatically calculated by the Vevo 2100 standard measurement package.

**Detection for renal function parameters**

Blood samples were obtained from the tail veins and collected into non-heparinized tubes 48 h after reperfusion. Centrifugations of blood samples were performed and the sera were stored at -20°C for the blood urea nitrogen (BUN) and serum creatinine (Scr) determinations by an Auto Analyzer with BUN and Scr test kits (BioSystems, Shanghai, China). Kidneys were removed and homogenized with ice-cold saline to prepare a 40% homogenates which were used for the determinations of GSH by spectrophotometric technique reported by Richardson and Murphy [31] and NO using colorimetric Griess reaction [32].

**Histology**

Kidneys were removed and washed in PBS buffer. The samples were cut into 2.5 mm blocks and immersed in 10% formaldehyde and then prepared for hematoxylin-eosin (HE) staining. The blocks were dehydrated in ethanol and xylene and then embedded in paraffin. One 4 μm section was cut from a paraffin block and stained with HE to determine renal tubular injury. For quantification of tubular injury score, sections were assessed by counting the percentage of tubular necrosis using the following scoring criteria [33]: 0 = normal, 1= <10%, 2 = 10% to 25%, 3 = 26% to 50%, 4 = 51% to 75%, and 5 = >75%. Five to eight samples were assessed in each group. Five sections were selected for each sample and two non-duplicate high power fields (HPFs) (400 × magnification) were selected for each section.

**Isolated renal artery preparation as a model for hypoxia/reoxygenation**

Mice were killed quickly by cervical dislocation and the renal arteries were dissected out and then transferred to a petri dish containing Krebs’ solution. The renal artery was trimmed free of connective tissue and cut into 2 mm segments in length and mounted in a 50 ml organ bath and connected to an isometric force displacement transducer (MLT0202P N8 041.3008, AD Instruments, Australia). The transducer output was amplified and recorded using the Mac Laboratory recording system (AD Instruments, Australia). Hypoxia/reoxygenation was induced by submitting the renal artery to 20 min of hypoxia by changing the gas mixture to 95% N₂ and 5% CO₂, followed by 30 min of reoxygenation by changing the gas mixture back to 95% O₂ and 5% CO₂. The effect of hypoxia/reoxygenation on vascular reactivity was examined on the dose response curve of acetylcholine (ACh) and sodium nitroprusside (SNP) of the renal artery. Vitamin C (10⁻² M) was administered to investigate the effect on vascular reactivity. A time-matched control was carried out parallel to each experiment.

**Western blot**

Protein lysate was collected and resolved on SDS-PAGE. After transfer to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), the blots were blocked in TBST(1mM CaCl₂, 136mM NaCl, 2.5mM KCl, 25mM Tris–HCl, 0.1% Tween 20) containing 5% nonfat dry milk and incubated with specific primary antibodies and corresponding secondary antibodies. Rabbit anti-superoxide dismutase (SOD) antibody was purchased from Cell Signaling Technology (1:1,000). Secondary anti-rabbit HRP-linked antibody was purchased from Cell Signaling Technology (1:1,000). GAPDH (1:2,000, Cell Signaling Technology, USA) was used as loading control. Bands were visualized by Bio-Rad Molecular Imager ChemiDoc XRS plus System.
Fig. 1. Tubular injury 48 h after ischemia/reperfusion. A, B, C and D were the representative sections from Con, IRI, Con+VC, IRI+VC, respectively. Kidneys from Con and Con+VC groups showed normal tubular structure without any sign of injury. IRI group showed significant tubular necrosis and degradation but vitamin C administration ameliorated the tubular damage (Hematoxylin and Eosin Staining ×400). Con: control group, IRI: Ischemia/reperfusion injury group, Con + VC: control group in presence of vitamin C, IRI + VC: Ischemia/reperfusion injury in presence of vitamin C.

ROS detection
ROS level was estimated using Total ROS Activity Assay Kit (eEnzyme, USA). Fresh renal arteries were homogenized in ice-cold isolation buffer with a Kontes-Duall glass homogenizer. The homogenate was centrifuged at 1,700 × g for 5 min at 4°C to pellet tissue debris and nuclei. The whole supernatant lysate was used for detection of tissue total ROS. Samples were incubated with Total ROS Green in a 5% CO₂, 37°C incubator for 6 hours. Fluorescence intensity was monitored at 490 nm excitation and 525 nm emission using fluorescence plate reader with bottom read mode (SpectraMax M3, Molecular Devices).

Statistical Analysis
All data were expressed as mean ± SD. One-way ANOVA was used to analyze the data among four groups. Independent t-test was performed for comparison between two groups. Difference was considered statistically significant when \( P < 0.05 \). All statistical tests were performed using SPSS software package 20.0 for Windows.

Results

Effect of vitamin C on tubular morphology following ischemic injury
Renal ischemia/reperfusion is well-known to mainly cause tubule damage. We focused on the changes in tubular histology after reperfusion. The kidney sections from Con group revealed the normal renal structure and tubules without any signs of necrosis (Figure 1A). IRI led to significant tubular necrosis and degradation 48 h after the onset of reperfusion (Figure 1B). The tubular injury scores were 0.2 ± 0.2 and 4.2 ± 0.2 for Con group and IRI group, respectively \(( P < 0.05 )\). The kidney sections in Con+VC group showed the same histological structure to Con group and there was no signs of cell swelling and necrosis (injury score: 0.4 ± 0.3 vs 0.2 ± 0.2, \( P = 0.6 \), Figure 1C). Vitamin C supplementation reduced the severity of tubular injury following ischemic reperfusion (injury score: 2.8 ± 0.4 vs 4.2 ± 0.2, \( P < 0.05 \), Figure 1D).
Fig. 2. BUN and Scr levels were detected using vein blood samples while GSH and NO levels were measured in kidney lysate. Vitamin C supplementations for IRI + VC groups caused reduction in BUN and Scr levels (panel A and B) and elevated GSH and NO levels (panel C and D) compared with IRI groups. There were no significant differences in BUN, Scr, GSH and NO levels between Con and Con + VC groups. BUN: blood urea nitrogen, Scr: serum creatinine, GSH: reduced glutathione, NO: nitric oxide, Con: control group, IRI: Ischemia/reperfusion injury group, Con + VC: control group in presence of vitamin C, IRI + VC: Ischemia/reperfusion injury in presence of vitamin C. *P < 0.05 for IRI vs Con, △P < 0.05 for IRI+VC vs IRI.

Effect of vitamin C on renal function parameters following ischemic injury

Vitamin C supplementation significantly reduced BUN and Scr levels 48 h after reperfusion compared to the IRI group (128.8±24.4 vs 69.2±29.4 mg/dl; 0.91±0.06 vs 0.76±0.05 mg/dl; P<0.05, Figure 2A and 2B). In addition, Pretreatment with vitamin C for mice subjected to ischemia/reperfusion significantly elevated renal GSH and NO levels at 48 h after reperfusion compared with the IRI group (2.6±0.21 vs 3.4±0.36 ug/mg protein; 0.42±0.02 vs 0.93±0.06 nmol/mg protein; P<0.05, Figure 2C and 2D).

In vivo renal arterial resistance and oxidative stress

We found that IRI affected renal arterial resistance and the renal arterial resistance index was significantly increased in IRI mice compared with control mice (0.68±0.05 vs 0.56±0.03, P<0.05, Figure 3A). Vitamin C administration decreased renal arterial resistance index of IRI mice (0.57±0.04 vs 0.68±0.05, P<0.05, Figure 3A).

IRI caused more than two-fold increase in total ROS level in renal artery (2.3±0.3 vs 1.0±0.2 F/F0, P<0.05, Figure 3B). Vitamin C administration decreased ROS level of renal artery in IRI mice (1.6±0.2 vs 2.3±0.3 F/F0, P<0.05, Figure 3B). The SOD expression was decreased in renal artery of IRI mice compared with control mice (0.25±0.04 vs 1.0±0.08, P<0.05, Figure 3B). Vitamin C supplementation preserved the SOD expression following ischemia/reperfusion (0.52±0.06 vs 0.25±0.04, P<0.05, Figure 3B).
In vitro vascular reactivity of renal artery subjected to hypoxia/reoxygenation

Vitamin C significantly potentiated the ACh-induced relaxation in the Con rings (Figure 4A). It was also noted that vitamin C significantly potentiated the ACh-induced relaxations of the renal rings subjected to hypoxia/reoxygenation at all doses in vitro study (left panel in Figure 4A). In presence of vitamin C, 2 μM ACh increased hypoxic renal arterial relaxation by 14±2.3% which was similar to the elevation in renal arterial relaxation induced by the same dose of ACh in Con rings with the presence of vitamin C (right panel in Figure 4A). Hypoxia followed by reoxygenation significantly impaired the SNP-induced relaxations at all doses but vitamin C significantly potentiated the SNP-induced relaxations in the renal arterial rings submitted to hypoxia/reoxygenation at all doses (left panel in Figure 4B). SNP-induced relaxation (10^{-8} M) reached 23±1.1% in the hypoxic rings compared to 34±1.3% in the control rings (P<0.05, right panel in Figure 4B). Vitamin C also potentiated the SNP-induced relaxation in Con rings (right panel in Figure 4B).

Fig. 3. Effects of vitamin C on renal arterial resistance and oxidative stress. A. Renal arterial pulse wave Doppler. IRI caused changes in renal arterial pulse wave pattern. Renal artery peak systolic velocity was increased while renal artery end-diastolic velocity was decreased in IRI group. IRI induced an increase in renal arterial resistance index but vitamin C attenuated the increased renal arterial resistance index. Renal arterial resistance index was calculated as (renal artery peak systolic velocity - renal artery end-diastolic velocity) / renal artery peak systolic velocity. B. ROS level and SOD expression in renal arteries. IRI significantly increased ROS level and decreased SOD expression. Meanwhile, vitamin C partly preserved the ROS level and SOD expression following IRI. F0 was defined as mean of fluorescence value of Con group. Con group was used as calibrator sample. Con: control group, IRI: Ischemia/reperfusion injury group, Con + VC: control group in presence of vitamin C, IRI + VC: Ischemia/reperfusion injury in presence of vitamin C. *P < 0.05 for IRI vs Con, △P < 0.05 for IRI+VC vs IRI.
Discussion

In the current study, the roles of vitamin C in the modulation of kidney function and renal arterial reactivity after ischemic injury have been investigated. We found that vitamin C supplementation improved the renal function as evidenced by reduced levels of Scr and BUN and increased NO production and GSH level. The tubular injury scores decreased from 4.2 ± 0.2 in IRI group to 2.8 ± 0.4 in IRI + VC group and vitamin C supplementation caused a noticeable reduction in the severity of renal tubular injury. Moreover, vitamin C supplementation decreased resistance index of renal artery and alleviated oxidative stress status secondary to IRI as shown by significant reduction in renal arterial ROS level and elevation in renal arterial SOD expression. Pretreatment with vitamin C significantly potentiated the Ach-induced relaxations in both Con and Hypo rings. SNP-induced relaxations in Con and Hypo rings were also potentiated by vitamin C.

Fig. 4. Effect of vitamin C on the relaxation of renal arterial rings exposed to hypoxia/reoxygenation in vitro. Hypoxia followed by reoxygenation significantly impaired the ACh-induced relaxations at all tested doses and vitamin C significantly potentiated ACh-induced relaxations in the renal arterial rings exposed to hypoxia/reoxygenation process (panel A). Hypoxia followed by reoxygenation significantly impaired the SNP-induced relaxations at all tested doses and vitamin C significantly potentiated SNP-induced relaxations in the renal arterial rings exposed to hypoxia/reoxygenation process (panel B). Ach: acetylcholine, SNP: nitroprusside, Con: control group, Hypo: hypoxia/reoxygenation group, Con + VC: control group in presence of vitamin C, Hypo + VC: hypoxia/reoxygenation in presence of vitamin C. *P < 0.05 for IRI vs Con, #P < 0.05 for IRI+VC vs IRI.
Both NO and GSH as oxidative stress indicators are involved in renal ischemic injury. NO could diminish leukocyte adhesion, neutrophil infiltration and the formation of inflammatory mediators for the tissues underwent ischemia/reperfusion process [34]. Kurata et al. found that pretreatment with FK409, a NO donor, attenuated ischemia/reperfusion induced renal dysfunction, histological damage and endothelin-1 (ET-1) overproduction whereas non-selective NO synthase inhibitor L-NAME administration aggravated renal injury, which suggests that the suppressive effect of NO on renal ET-1 overproduction induced by IRI may be probably responsible for the protective function of NO against ischemic acute renal injury [35]. The further study demonstrated that the suppression of NF-kB by FK409 was involved in the FK409-induced inhibition of ET-1 production and ameliorated kidney dysfunction in IRI model [36]. In this study we found that NO level was significant decreased after IRI but NO level and renal function were improved by vitamin C supplementation. Our results are in agreement with previous reports demonstrating that vitamin C has protective effects on tissues with IRI [22, 37-39]. In addition, our findings also showed that vitamin C administration elevated GSH level after renal ischemic injury. GSH with glutathione peroxidase (GPx) metabolizes hydrogen peroxide and organic hydroperoxides to hinder the peroxidation chain reaction and protect protein thiol groups from non-enzymatic oxidation [19]. There are lots of studies revealing that GSH level was significantly decreased in different IRI models [20, 40-42].

There is urgent need to explain how vitamin C influences NO and GSH levels since the current results suggest that the beneficial effect of vitamin C might be related to the elevations in NO and GSH levels. The generated superoxide is converted into hydrogen peroxide firstly and then hydrogen peroxide is metabolized into water and oxygen by catalase and/or GPx during which a large amount of GSH will be consumed [43]. The possible explanation is that vitamin C may preserve GSH level via scavenging superoxide effectively. The free radical $O_2^-$ at high level will react with NO to produce peroxynitrite [27]. There is a possibility that vitamin C restores NO level by reducing $O_2^-$. Moreover, our study indicated that vitamin C ameliorated the increased renal arterial resistance following renal ischemic injury in vivo and also promoted the relaxation of hypoxic renal arterial rings in vitro. Vitamin C supplementation reduced renal arterial ROS level in IRI model. Phillips et al. reported that increased vascular superoxide production impaired vessel dilation to Ach but the treatment of vessels with the free radical scavenger tempol improved dilation to Ach [44]. The study by Drew et al. showed that oxidative stress promoted renal vascular resistance and renal vasoconstriction during exercise in patients with peripheral arterial disease but ascorbic acid, also known as vitamin C, attenuated the augmented renal vascular resistance [45]. It is well known that the enhanced resistance of renal artery is linked to reduction in renal blood flow which may also contribute to kidney damage after IRI. Thus, vitamin C might protect renal vascular function via reducing ROS level and further improve kidney function in IRI. It should be noted that except for ROS, reactive nitrogen species (RNS) such as peroxynitrite also markedly aggravates oxidative stress injury [27]. Excessive peroxynitrite which is generated by the reaction between NO and elevated $O_2^-$ could be critically instrumental in the vasoconstriction [46]. So vitamin C is likely to scavenge peroxynitrite to preserve renal vascular relaxation. In our study, the SOD expression was increased by the vitamin C administration in IRI. SOD constitutes the first line of defence against ROS and it is very crucial for the removal of $O_2^-$. There are lots of studies revealing that IRI induces a reduction in SOD activity [49-51]. Although it is unclear that how vitamin C upregulates SOD specifically, increased SOD expression would certainly eliminate superfluous oxygen free radical and protect against oxidative stress damage [47]. The protective roles of vitamin C in renal vascular reactivity and kidney function in IRI may involve the regulation of SOD.
Conclusion

This study supports that vitamin C attenuates renal ischemic injury and ameliorates renal arterial resistance and renal arterial reactivity. The mechanisms for the protective roles of vitamin C in IRI may involve the preservation of NO, GSH, and SOD levels.

Disclosure Statement

None.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No. 81371443andNo.81400305),Beijing NaturalScience Foundation(No.7152045, No.7122056, No.7142049 and No.7142137), Beijing Talents Fund (No.2014000021469G233), Beijing Municipal Administration of Hospitals Incubating Program (No.PX2016046), Basic and Clinical Research Foundation of Capital Medical University (No.13JL26), Natural Science Foundation of Xinjiang (No.2014211A063) and Beijing Municipal Health Bureau High-Level Talent Cultivation (No.2014-3-043).

References

Zhu et al.: Vitamin C and Kidney


31 Richardson RJ, Murphy SD: Effect of glutathione depletion on tissue deposition of methylmercury in rats. Toxicol Appl Pharmacol 1975;31:505-519.


