*J Vet Clin* 25(3) : 165-169 (2008)

Journal of Veterinary Clinics
www.ksvc.or.kr

# Antioxidant Effects of Ascorbic Acid on Renal-Ischemia Reperfusion Injury in Rabbit Model

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(Accepted: June 5, 2008)

**Abstract :** Renal ischemia-reperfusion (I/R) injury is great clinical important because viability of the organ depends on the tolerance to ischemia-reperfusion injury, an inevitable processing during surgery. The purpose of this study was to investigate the effects of premedicated ascorbic acid alone in I/R injury model induced by cross-clamping of renal vessels. In the rabbit models, 2-4 kg New Zealand white rabbits were subjected to 30 minutes of warm unilateral renal ischemia followed by removal of contralateral kidney and then divided into five groups, control (2) and treatment groups (3). In control group 1, the rabbits only received right nephrectomy. In control group 2, the rabbits received I/R on left kidney after the right nephrectomy. In treatment group 1, the rabbits received ascorbic acid 50 mg/kg IV before the operation. In treatment group 2, the rabbits received ascorbic acid 100 mg/kg IV before the operation. In treatment group 3, the rabbits received ascorbic acid 200 mg/kg IV before the operation. Blood samples were collected from these rabbits for measurement of kidney function tests at the 0, 1st, 3rd and 7th day and antioxidant enzyme( SOD, GSHPx, CAT) at 24 hours. Kidney function tests (serum creatinine and BUN) showed a significant difference between group 2 and group 4, 5. Activity of antioxidant enzymes in plasma were significant decrease in group 4, 5 compare to group 2. The result of this study suggested that the exogenous ascorbic acid had a role of attenuation of renal I/R injury in rabbit model.

***Key words*** **:** ascorbic acid, rabbit, antioxidant, renal ischemia-reperfusion.

## Introduction

Tissue subjected to a period of ischemia undergoes morphological and functional damages, which increase during the reperfusion phase (14). Ischemia-reperfusion (I/R) injury in the kidney is often observed in the renal operation. Thus, to decrease of tissue damage is important to ameliorate cause of renal cell death, renal failure and delayed graft function. Reperfusion of ischemic kidneys increases the hazardous effect of early ischemic injury by release of reactive oxygen species (ROS) and accumulation of activated neutrophils (7). In addition, ROS cause lipid peroxidation of cellular membranes and, hence, disruption of the structural integrity and capacity for cell transport and energy production (4).

Renal I/R injury leads to production of excessive amounts of ROS and reactive nitrogen species (RNS), causing oxidative stress which results in alterations in mitochondrial oxidative phosphorylation, depletion of ATP, an increase in intracellular calcium and activation of protein kinases, phosphatases, proteases, lipases and nucleases leading to loss of cellular function and integrity (24). Evidence of oxygen radicalmediated injury in kidney includes demonstration of renal injury being accentuated by oxidants and the observation that deficiency of antioxidants exacerbates renal injury and that free radicalmediated lipid peroxidation occurs as a manifestation of ischemiareperfusion injury also implicate oxidants in the pathophysiology of acute renal failure (15). Therefore, operated patient may not lead to better outcome if did not reduce these hazardous metabolites.

To reduce these metabolites, many investigators has been examined the variety of scavenger for the free radical oxygen. That is the effects of external supplementation of antioxidants (12) and the activities of endogenous enzymatic antioxidant defense system in kidney ischemia/reperfusion injury (10). The reason is that kidneys have enzymatic [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)] and nonenzymatic (tocopherols, carotenes, ubiquinol, glutathione and ascorbic acid) antioxidant defenses to cope with this potential damage (13). Therefore, the protective effect of supplementation with antioxidant against ischemiareperfusion induced oxidative stress provides unequivocal evidence that antioxidant enzymes impact on the degree of tissue damage.

Nonenzymatic antioxidant, ascorbic acid is the primary watersoluble antioxidant in human plasma, capable of scavenging oxygenderived free radicals and sparing other endog-

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enous antioxidants from consumption (11,21). Additionally, ascorbic acid was used to protect against corneal damage by free radicals in rabbits (22), and also improved renal hemodynamics and decreased oxidative stress, inflammation, and fibrosis in the porcine ischemic kidney (8). Although compound form of vitamin C and E and other antioxidants were used for attenuation of renal ischemiareperfusion injury in many studies, single dosage of ascorbic acid was only reported in experiment related with I/R injury in the canine (16,17).

The purpose of the present study is to clarify the effects of ascorbic acid and to determine the effective dose of ascorbic acid on renal I/R injury induced by cross clamping of renal vessels in rabbits.

## Materials and methods

### 1. Animals

Fifteen New Zealand white rabbits weighing 2-4 kg were used for the experiments. These animals were acclimated and maintained on a standard diet for 4 weeks and were demonstrated normal renal function before the surgical procedure.

### 2. Experimental groups

1) Control group 1 (n = 3); only the right kidney was removed

2) Control group 2 (n = 3); After the right nephrectomy, the left kidney was freed from the perirenal tissue and fat. A bolus of 150 IU/kg of heparin was given IV 3 minutes before ischemia and the left renal vessels were clamped with an atraumatic vascular clamp. After ischemia for 30 minutes, the clamp was removed and the blood reflows.

3) Treatment group 1 (n = 3); ascorbic acid 50 mg/kg IV before the operation. The left kidney was freed from the perirenal tissue and fat. And then, left renal artery and vein were clamped with an atraumatic vascular clamp. After ischemia during 30 minutes, then the renal vessels were unclamped. The right nephrectomy was performed.

4) Treatment group 2 (n = 3); ascorbic acid 100 mg/kg IV before the operation. The left kidney was freed from the perirenal tissue and fat. And then, left renal artery and vein were clamped with an atraumatic vascular clamp. After ischemia during 30 minutes, then the renal vessels were unclamped. The right nephrectomy was performed.

5) Treatment group 3 (n = 3); ascorbic acid 200 mg/kg IV before the operation. The left kidney was freed from the perirenal tissue and fat. And then, left renal artery and vein were clamped with an atraumatic vascular clamp. After ischemia during 30 minutes, then the renal vessels were unclamped. The right nephrectomy was performed.

### 3. Surgical procedure

After overnight fast, 30 minutes before induction of anesthesia, the animals were premedicated with atropine sulfate (Atropine Sulfate®, Huons Co., Korea, 0.04 mg/kg, SC), cefazolin sodium (Cefazolin®, Chong Kun Dang Co., Korea, 20 mg/kg, IV) and meloxicam (Metacam®, Boehringer Ingelheim Co., Korea, 0.2 mg/kg, IV). The animals were anesthetized with ketamine (Ketalar®, Yuhan Co., Korea, 85 mg/kg, IM) and Xylazine (Rumpun®, Bayer, Korea, 6 mg/kg, IM).Laparotomy was performed by midline incision. The left kidney was isolated, and then both the renal artery and vein were clamped. After 30 minutes of warm ischemia, the vessels were unclamped, and the right kidney was removed. Postoperatively, the rabbits were allowed free access to water and food.

### 4. Renal function

Blood urea nitrogen (BUN) and creatinine levels were determined on serum samples taken preoperation and at 1, 3 and 7 days after procedure from jugular vein, using a commercially available kit (VetTest 8008, IDEXX Co., USA). The results are expressed as milligrams per deciliter.

### 5. Antioxidant enzyme activity in plasma

Blood samples were collected preoperation and 24 hours after procedure using an anticoagulant as EDTA, and centrifuged at 700-1,000 × g for 10 minutes at 4°C Then, the samples were pipetted off the top yellow plasma layer without disturbing the white buffy layer, and collected plasma samples were stored plasma on ice until assaying or freeze at –80°C.

1) Superoxide dismutase (SOD) activity was determined with a commercial Superoxide Dismutase Assay Kit® (Cayman, Co., USA) for the measurement of SOD activity from plasma. SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format. The activity was recorded spectrophotometrically at 450 nm. The enzyme activity was calculated as U/ml.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

2) Glutathione peroxidase (GSHPx) activity was measured with a commercial Glutathione Peroxidase Assay Kit® (Cayman, Co., USA). The kit measures GSHPx activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of an organic hydroperoxide by GSHPx, is recycled to its reduced state by glutathione reductase and NADPH. The activity was recorded spectrophotometrically at 340 nm. The enzyme activity was calculated as nmol NADPH oxidized $min^{-1}ml^{-1}$.

$$R\text{-}O\text{-}O\text{-}H + 2GSH \rightarrow R\text{-}O\text{-}H + GSSG + H_2O$$

3) Catalase (CAT) activity was measured with a commercial Catalase Assay Kit® (Cayman, Co., USA), utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of

$H_2O_2$. The activity was recorded spectrophotometrically at 540 nm. Catalase activity was calculated as nmol $H_2O_2$ consumed $min^{-1}ml^{-1}$.

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

### 6. Statistical analysis

All values are expressed as means ± SD of determinations for all dogs in the group. Data were analyzed using analysis of variance followed by twoway repeated measures analysis (ANOVA) and a *P* value below 0.05 was considered statistically significant.

## Results

### 1. Renal function

Serum creatinine levels, measured as an index of kidney function. The level of creatinine in the group 2 and 3 increased to 2.58 mg/dl, 2.48 mg/dl at the first day after operation. And then, the levels were getting down normal range. At the first and 3rd day, group 2 and 3 were keeping the higher level than other groups. However, The levels of creatinine were significantly decreased in the group 4 and 5 at the fist and 3rd day after reperfusion compare to group 2. However, there was no significant difference between group 4 and 5 (Fig 1).

We also measured BUN as a second index of kidney function in these experimental groups. Similar to serum creatinine, the levels of BUN in the group 2 and 3 increased to 49.2 mg/dl, 43.3 mg/dl at the first day after reperfusion. The BUN levels in the other groups increased to 33.03 mg/dl (normal range, 7.0-27.0) after reperfusion and then gradually decreased to normal level. The levels of BUN were significantly decreased in the group 4 and 5 at the first, 3rd and 7th day after reperfusion compare to group 2. However, there was no significant difference between group 4 and 5 (Fig 2).

### 2 . Antioxidant enzyme activities in plasma

The activities of various antioxidant enzymes in plasma exposed to 30 minutes of ischemia followed by 24 h of reperfusion are shown as follows.

The levels of SOD activity (U/ml) was 8.95 ± 0.40 (group 1), 8.09 ± 0.06 (group 2), 8.18 ± 0.33 (group 3), 8.90 ± 0.41 (group 4), 8.92 ± 0.17 (group 5) followed by 24 h reperfusion. The SOD levels of group 2 were significant compared with preoperation($p < 0.05$). The levels of SOD were significantly increased in the group 4 and 5 at the 24 hours after reperfusion compare to group 2 (Fig 3).

The levels of GSHPx activities (n mol/min/ml) were measured. They were 143.20 ± 12.23 (group 1), 118.01 ± 2.81 (group 2), 125.268 ± 15.80 (group 3), 150.24 ± 13.77 (group 4) and 163.76 ± 13.28 (group 5) followed by 24 h reperfusion. The levels of GSHPx were significantly increased in the group 4 and 5 at the 24 hours after reperfusion compare to group 2 (Fig 4).

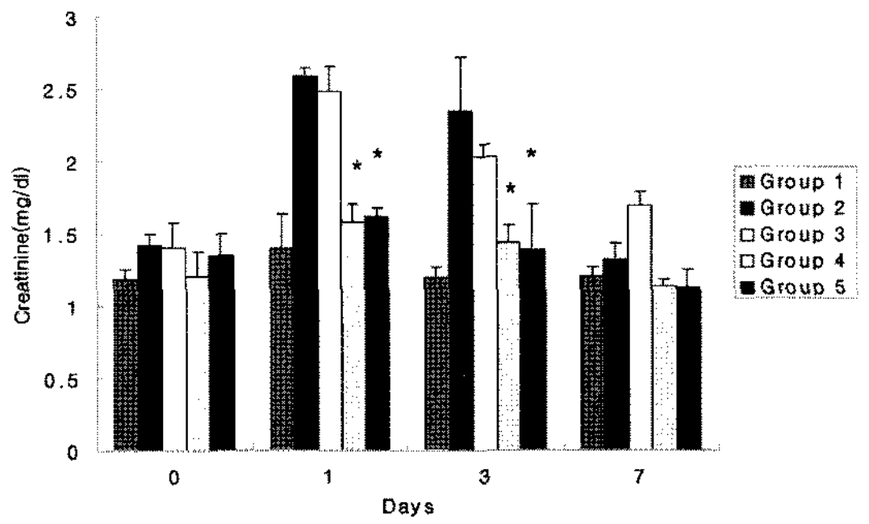


**Fig 1.** Serum creatinine levels in rabbits after 30 minutes of I/R. The values are expressed as mean ± SD for all groups. *$p < 0.05$ for group 4 and 5 versus group 2 at same day.

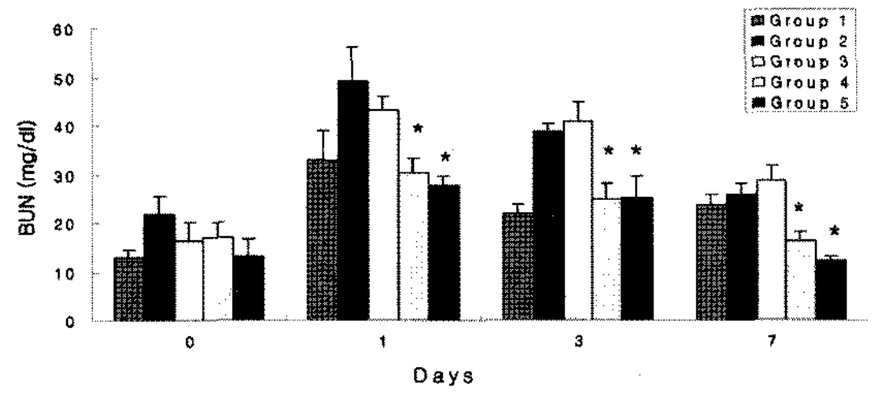


**Fig 2.** BUN levels in rabbits after 30 minutes of I/R. The values are expressed as mean ± SD for all groups. *$p < 0.05$ for group 4 and 5 versus group 2 at same day.

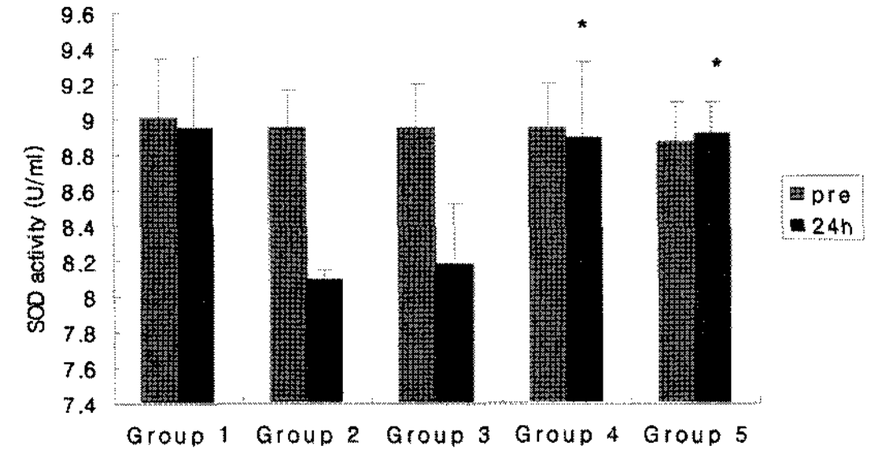


**Fig 3.** The activity of total SOD in plasma exposed to 30 min of ischemia followed by 24 h reperfusion. The values are expressed as mean ± SD for all groups. *$p < 0.05$ for group 4 and 5 versus group 2.

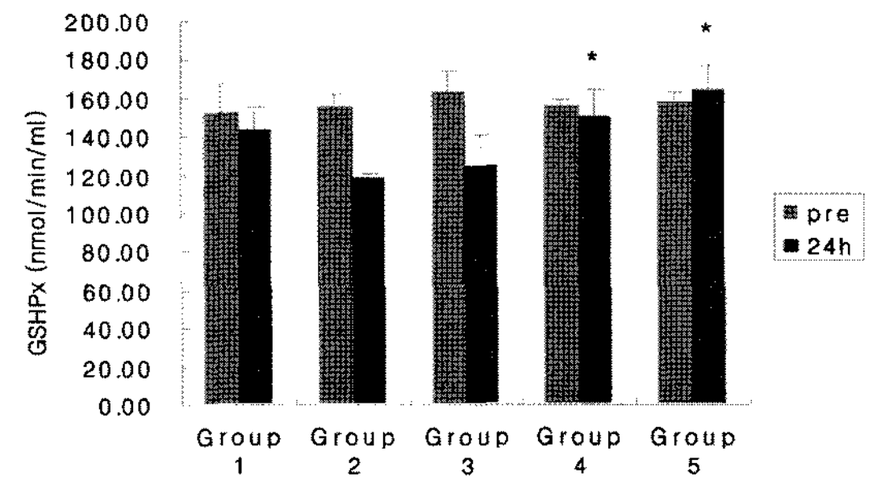


**Fig 4.** The activity of GSHPx in plasma exposed to 30 min of ischemia followed by 24 h reperfusion. The values are expressed as mean ± SD for all groups. *$p < 0.05$ for group 4 and 5 versus group 2.

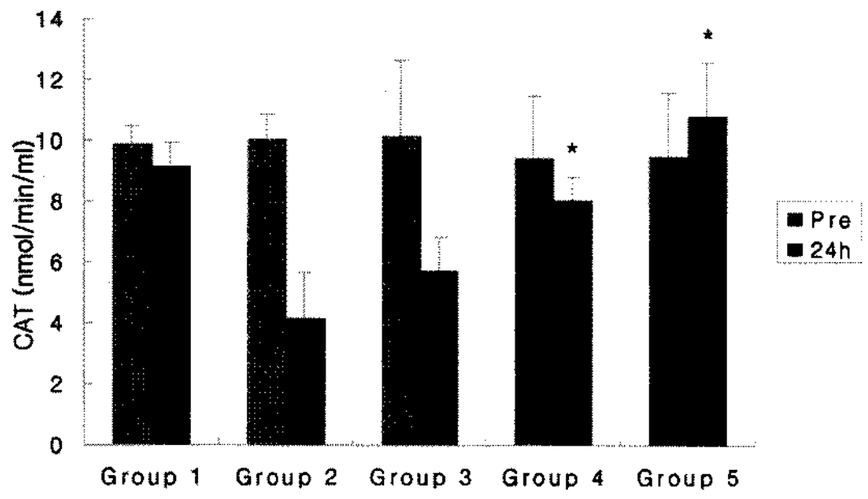


**Fig 5.** The activity of CAT in plasma exposed to 30 min of ischemia followed by 24 h reperfusion. The values are expressed as mean ± SD for all groups. *$p < 0.05$ for group 4 and 5 versus group 2.

The levels of CAT activities (n mol/min/ml) were measured. They were 9.19 ± 0.74 (group 1), 4.15 ± 1.49 (group 2), 5.74 ± 1.10 (group 3), 8.06 ± 0.77 (group 4) and 10.80 ± 1.79 (group 5) followed by 24 h reperfusion. The CAT levels of group 2 and 3 were significant compared to pre operation ($p < 0.05$). The levels of CAT were significantly increased in the group 4 and 5 at the 24 hours after reperfusion compare to group 2 (Fig 5).

## Discussion

A number of studies have performed to ameliorate of these I/R injuries (13,24). As well as ameliorator for I/R injury, studies for oxidative stress associated with ischemiareperfusion have investigated in rats (20), dogs (6), and rabbits (9). Oxidative stress represents the imbalance between oxidants such as ROS and antioxidants (25), and it probably contributes to the development, progression, and complication of acute renal failure as well as to chronic renal failure, which is characterized by the increased production or decreased elimination of antioxidants (1).

Ascorbic acid regenerates vitamin E, glutathione, and flavonoids (26). Therefore, it protects against free radical mediated protein inactivation associated with the oxidative burst of neutrophils. Also, ascorbic acid reduces reactive oxidant species intracellularly and extracellularly, and maintain transition metals in reduced form, and may quench free radical intermediates of carcinogen metabolism. Ascorbic acid is an outstandingly powerful antioxidant that reacts rapidly with a variety of oxidants, including the rather poorly reactive superoxide anion radical (19).

Renal function was evaluated by BUN and serum creatinine levels in this study. The levels of BUN and creatinine were significantly increased in group 2 and 3 at the first and 3rd day after reperfusion. And the levels of BUN and creatinine were significantly decreased in the ascorbic acid treated group 4 and 5 at the first, 3rd and 7th day after reperfusion compare to group 2. This result was similar to the previous report (16) that administration of ascorbic acid for renal I/R injury decreased blood BUN level in the canine. However, there was no significant difference between ascorbic acid treated group 4 (100 mg/kg) and group 5 (200 mg/kg). These results suggested that renal blood flow was continued by reperfusion or tolerance for the ischemia was increased as condition of ischemic preconditioning. The mechanisms of ischemic preconditioning seem to include a few welldescribed signal transduction pathways. These mechanisms include adenosine receptor mediated activation of adenosine triphosphategated potassium channels (19), nitric oxide synthesis (1), free radical generation (2), and the upregulation of molecular chaperones (23).

Present study suggest that antioxidant enzymes (SOD, GSHPx, and CAT) levels, which protect oxygen free radical, increased in ascorbic acid treated group. This result was similar to the previous report (17) that administration of ascorbic acid for renal I/R injury increased antioxidant enzymes (SOD, GSHPx, and CAT) levels in the canine. Generally, the conversion of superoxide anion and hydrogen peroxide was impaired due to decreased levels of SOD, GSHPx and CAT, resulting in the increase of oxygen free radicals(18). Thus, the elevated superoxide and hydrogen peroxide levels accelerate the damage to the kidney. However, ascorbic acid as antioxidant detoxified hydrogen peroxide. Reactive oxygen species are normal byproducts of cellular metabolism and are detoxified by the antioxidant capacity of the cell. This delicate balance between the enzyme systems that produce ROS and the antioxidant enzymes that detoxify ROS is critical for normal structure or function of cell and organ. Recognition of the contribution of ROS to the pathophysiology of injury observed in ischemiareperfusion (3) had resulted in the utilization of exogenous antioxidant enzymes as potential therapeutic agents to neutralize the excessively produced oxygen derived free radicals, but with limited success (5). A possible explanation for the limited success may be the inability of these antioxidant enzymes to gain access to the site of injury as these proteins are large molecules. In this study, ascorbic acid as exogenous antioxidant was considered that it had ability for attenuation of I/R injury through increases the activities of SOD, GSHPx and CAT. On the other hand, concentration of antioxidant enzymes has increased for reduction of consumption rather than decreased due to another scavenger.

SOD, GSHPx and CAT are antioxidant enzymes participating in the detoxifying process of superoxide radicals and hydrogen peroxide in subsequent reaction. We investigated the enzymatic antioxidant status of rabbit kidneys after I/R. I/R causes a decrease in enzyme activity, enzyme protein and mRNA levels of catalase, GSHPx and CuZnSOD (10), however, it was supposed that the observed increase in total SOD, GSHPx and CAT following 24 h reperfusion might be caused by the increase in transcriptional activation of genes. Specially, it revealed more increase in the treatment group which was administrated ascorbic acid as antioxidant.

## Acknowledgements

This work was supported by grant No. R11-2002-100-04006-0 from ERC program of the Korea Science & Engineering Foundation.

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