J Vet Clin 27(5) : 553-558 (2010)

Journal of Veterinary Clinics
www.ksvc.or.kr

Attenuation of Renal Ischemia-Reperfusion (I/R) Injury by Ascorbic Acid in the Canine Nephrotomy

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(Accepted: September 27, 2010)

Abstract : The purpose of this study was to investigate the effects of premedicated ascorbic acid and hepa-saline irrigation/aspiration on attenuation of ischemia-reperfusion (I/R) injury and recovery of renal function in canine nephrotomy model. In the canine model, nine mixed dogs were subjected to renal nephrotomy with premedicated ascorbic acid and hepa-saline irrigation-aspiration (treatment group 2), and only hepa-saline irrigation-aspiration (treatment group 1). The level of renal function and antioxidant enzymes after nephrotomy were measured. And the expression pattern of TNF-α and INF-γ was examined in the renal tissue at 7th day after nephrotomy. BUN and creatinine levels significantly decreased in the treatment group 1 and 2 compared to that of control group at the 3rd, 5th and 7th day after reperfusion ($p < 0.05$). And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion ($p < 0.05$). The activities of antioxidant enzymes in plasma was significantly increased in the treatment group 1 and 2 compared to that of control group at the 3rd, 5th and 7th day after reperfusion ($p < 0.05$). And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion ($p < 0.05$). TNF-α was decreased and INF-γ was increased in treatment groups. The result of this study suggested that irrigation-aspiration has effects on attenuation of renal ischemia-reperfusion injury, and the exogenous ascorbic acid has a role in the attenuation of renal ischemia-reperfusion injury and recovery of renal function in canine nephrotomy model.

Key words **:** antioxidants, nephrotomy, ascorbic acid, dog, renal ischemia-reperfusion.

Introduction

Nephrotomy is usually performed not only to remove calculi lodged in the renal pelvis, but also to explore in the renal pelvis for neoplasia or hematuria. Approximately 4% of all urinary calculi in dogs occur in the kidney (5). Unfortunately, most of them are calcium oxalate, for which medical therapy is often ineffective (7). Nephrotomy is indicated for removal of stones from the renal pelvis that have not caused enlargement of the renal pelvis and proximal ureter beyond the concave surface of the kidney. Nephrotomy temporarily decreases renal function by 20% to 50% (15).

Tissue subjected to a period of ischemia undergoes morphological and functional damage, which increase during the reperfusion phase (21). Ischemia-reperfusion (I/R) injury in the kidney is often observed in the renal operation. Thus, to decrease of the degree in 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 (8). In addition, ROS cause lipid peroxidation of cellular membranes and, hence, disruption of the structural integrity and capacity for cell transport and energy production (3).

A number of mechanisms explain tissue I/R injury. In addition to reduced glomerular filtration and accumulation of leukocytes (20), ROS, reactive nitrogen species (RNS) generation, and the loss of antioxidant defense are also considered to play key roles. Many of these reactive molecules activate the signaling mechanisms that culminate in tumor necrosis factor (TNF) production (12). TNF-alpha is a proinflammatory cytokine capable of upregulating its own expression, as well as the expression of other genes important in the inflammatory response (14). IFN-gamma is produced by T cells and NK cells following stimulation with IL-12 and / or IL-18 and is upregulated during renal ischemia/reperfusion injury (10).

Vitamin C is a potent aqueous phase antioxidant that has been shown to improve endothelial dysfunction due to the interaction of endothelium-derived NO and ROS (33). Thus, a number of clinical studies showed that intravenous infusion of vitamin C or other antioxidants significantly reduces blood pressure in hypertensive patients (16). Vitamin C is administered both orally and intravenously to dogs to improve health and performance, despite the fact that this species is able to synthesis the vitamin (34). Such an antioxidant as vitamin C

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has been used for diminution of free radical oxygen followed by I/R injury of variety organs.

Many studies investigated effects of antioxidants in the I/R injury of an organ for the diminution of impairment by oxidative stress (23,31). Although previous studies were performed after renal ischemia by the cross-clamping of renal vessel or renal transplantation, the author thought that irrigation-aspiration may play a role in reducing of free radicals by a physical mechanism of free radical washing.

The purpose of this study is to clarify the effect of premedicated ascorbic acid alone before operation for reducing I/R injury. And through the model, author examined the effect of irrigation-aspiration in the same condition as the canine nephrotomy.

Materials and Methods

Animals

Both sexes, adult dogs weighing 4-7 kg were used in this study. These animals were acclimatized and maintained on a standard diet, routine lighting cycle and room temperature for 6 month. They had normal renal function before surgical procedure.

Experimental groups

The dogs were assigned randomly into a control group (n = 3) or two ascorbic acid treatment groups (n = 3)

Control group (n = 3)

The left kidney is freed from the perirenal tissue and fat. A bolus of 150 IU/kg of heparin is given IV on 3 minutes before ischemia and the left renal vessels are clamped with atraumatic vascular clamps. After ischemia for 20 minutes and neprotomy, the clamp is removed and the blood reflows. The right nephrectomy is also performed.

Treatment group 1 (n = 3)

The left kidney is freed from the perirenal tissue and fat. A bolus of 150 IU/kg of heparin is given IV on 3 minutes before ischemia and the left renal vessels are clamped with atraumatic vascular clamps. During ischemia for 20 minutes and procedure nephrotomy, heparinized saline is irrigated through the renal artery (50 mmHg) and aspirated the fluid from the renal vein, and then the renal vessels are unclamped. The right nephrectomy is also performed.

Treatment group 2 (n = 3)

Ascorbic acid 100 mg/kg IV before operation. The left kidney is freed from the perirenal tissue and fat. A bolus of 150 IU/kg of heparin is given IV on 3 minutes before ischemia and the left renal vessels are clamped with atraumatic vascular clamps. During ischemia for 20 minutes and procedure nephrotomy, heparinized saline is irrigated through the renal artery (50 mmHg) and aspirated the fluid from the renal vein, then the renal vessels are unclamped. The right nephrectomy is also performed.

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 are induced with thiopental sodium (Thionyl® Dai han Pharm. Co., Korea, 12.5 mg/kg, IV) and then maintained during the procedure with isoflurane 2% and 100% oxygen supply. Laparotomy was performed by midline incision. The left kidney was isolated, and then both the renal artery and vein were clamped. After 20 minutes of warm ischemia and nephrotomy, the vessels were unclamped, and the right kidney was removed. And then irrigation-aspiration was performed in treatment two groups. During the operation, all dogs were administrated a balanced electrolyte solution (10 ml/kg/hr, IV). Postoperatively, the dogs were allowed free access to water and food.

Renal function

Blood urea nitrogen (BUN) and creatinine levels were determined on serum samples taken pre- operation and at 1, 3, 5 and 7 days after surgical procedure from jugular vein, using a commercially available kit (VetTest 8008, IDEXX Co., Japan). The results are expressed as milligrams per deciliter.

Antioxidant enzyme activity in plasma

Blood samples were collected pre- operation and at 1, 3, 5 and 7 days after surgical 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.

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

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 $min^{-1}ml^{-1}$.

$2H_2O_2 \rightarrow O_2 + 2H_2O$

TNF-alpha, INF-gamma

At the 7th day after surgical procedure, animals were euthanized and tissue samples from the left kidney were taken. The tissure samples were mixed with sample buffer (0.5M Tris-Cl, pH6.8, 10% SDS, 2-mercaptoethanl, 0.05% bromphenol blue). The protein samples were boiled at 100ºC for 4 min and cooled on ice. The denatured protein samples were separated by electrophoresis on 7.5 or 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose Hybond-P paper (Amersham Corp.). The residual binding sites were blocked by incubating the filters with 5% dry milk in phosphate-buffered saline and 0.05% Tween-20 for 1 h at room temperature. The filters were incubated with anti-TNF-a or anti-INF-r for 1 h with shaking. After washing twice with phosphate-buffered saline and 0.05% Tween 20, the blots were incubated with anti-dog IgG peroxidase conjugate (Amersham Corp.). The antigen antibody complexes were visualized by chemiluminescence (ECL detection system, Amersham Corp.).

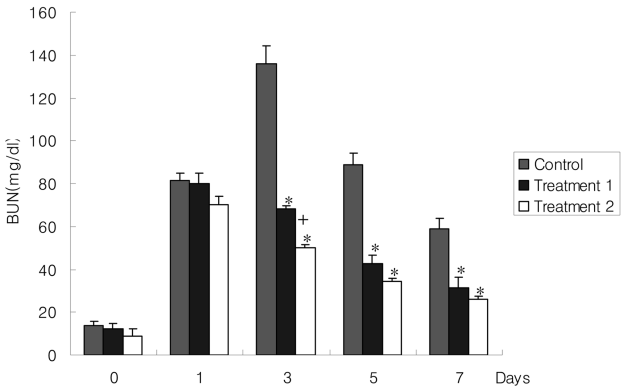

Fig 1. BUN levels in dogs after 20 minutes of I/R, expressed at the pre-operation and at the 1st, 3rd, 5th and 7th day after reperfusion. The values are expressed as mean ± SD for all groups. *p < 0.05 statistical significances compare to control group. +p < 0.05 statistical significances compare to treatment group 1.

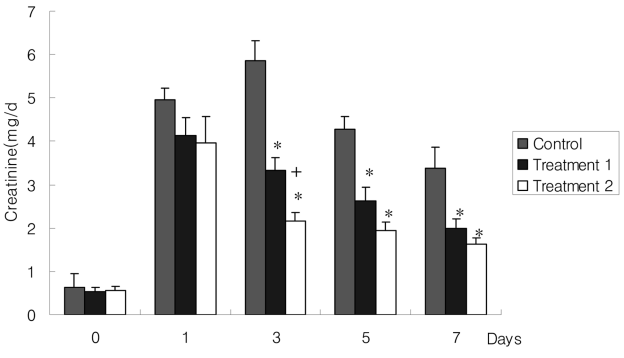

Fig 2. Creatinine levels in dogs after 20 minutes of I/R, expressed at the pre-operation and at the 1st, 3rd, 5th and 7th day after reperfusion. The values are expressed as mean ± SD for all groups. *p < 0.05 statistical significances compare to control group. +p < 0.05 statistical significances compare to treatment group 1.

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 two-way repeated measures analysis (ANOVA) followed by Student's *t*-test and a *P* value below 0.05 was considered statistically significant.

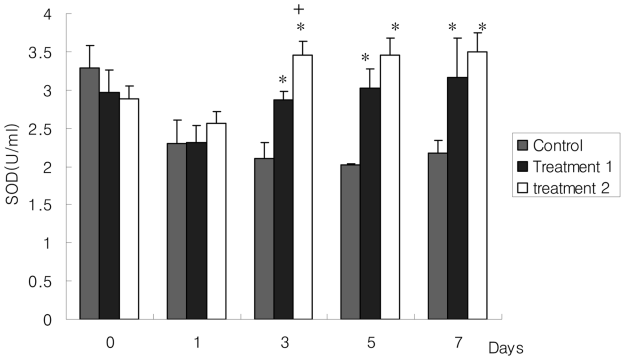

Fig 3. The activity of total plasma SOD in dogs after 20 minutes of I/R, expressed at the pre-operation and at the 1st, 3rd, 5th and 7th day after reperfusion. The values are expressed as mean ± SD for all groups. *p < 0.05 statistical significances compare to control group. +p < 0.05 statistical significances compare to treatment group 1.

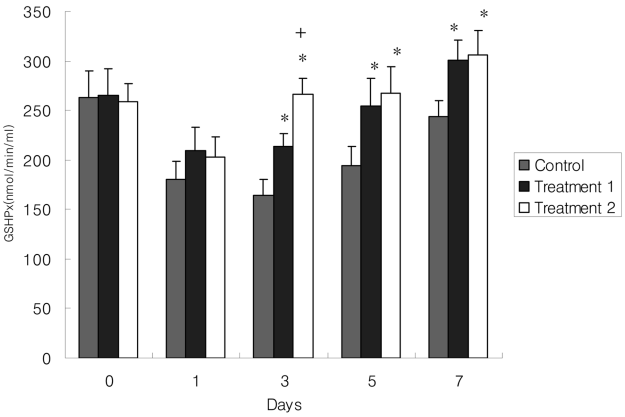

Fig 4. The activity of total plasma GSHPx in dogs after 20 minutes of I/R, expressed at the pre-operation and at the 1st, 3rd, 5th and 7th day after reperfusion. The values are expressed as mean ± SD for all groups. *p < 0.05 statistical significances compare to control group. +p < 0.05 statistical significances compare to treatment group 1.

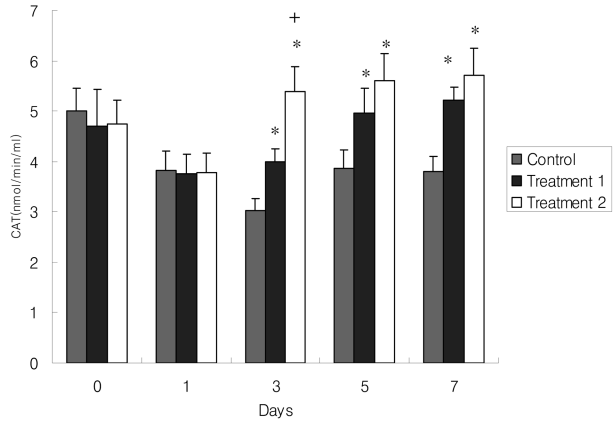

Fig 5. The activity of total plasma CAT in dogs after 20 minutes of I/R, expressed at the pre-operation and at the 1st, 3rd, 5th and 7th day after reperfusion. The values are expressed as mean ± SD for all groups. *$p < 0.05$ statistical significances compare to control group. +$p < 0.05$ statistical significances compare to treatment group 1.

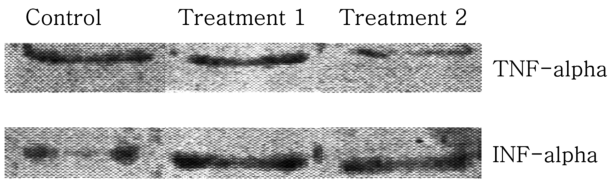

Fig 6. TNF-alpha, INF-gamma at the 7th day after nephrotomy.

Results

Renal function

The renal functions in plasma following renal nephrotomy were evaluated. The levels of BUN and creatinine were expressed at the pre-operation and at the 1st, 3rd, 5th and 7th day after reperfusion. The levels of BUN were significantly decreased in the treatment group 1 and 2 compare to control group at the 3rd, 5th and 7th day after reperfusion. And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion.

Antioxidant enzyme activity in plasma

The activities of antioxidant enzymes in plasma following nephrotomy were evaluated. The specific activities of SOD, GSHPx and CAT were expressed at the pre-operation and at the 3rd, 5th and 7th day after reperfusion. The activity of SOD was significantly increased in the treatment group 1 and 2 compare to control group at the 3rd, 5th and 7th day after reperfusion. And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion.

The activity of GSHPx was significantly increased in the treatment group 1 and 2 compare to control group at the 3rd, 5th and 7th day after reperfusion. And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion.

The activity of CAT was significantly increased in the treatment group 1 and 2 compare to control group at the 3rd, 5th and 7th day after reperfusion. And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion.

TNF-alpha and INF-gamma

The levels of TNF-alpha were decreased in treatment group 2 compared to those of other groups. The levels of INF-gamma were increased in treatment group 2 compared to those of other groups.

Discussion

When nephrotomy is usually performed to remove calculi, renal I/R injury is invariably followed acute renal failure. Thus, a number of studies have performed to ameliorate of these I/R injuries (17,22). As well as ameliorator for I/R injury, studies for oxidative stress associated with ischemia-reperfusion were investigated in rats, dogs and rabbits (4,11,26). Oxidative stress represents the imbalance between oxidants such as ROS and antioxidants (32), 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). We chose 20 minutes of warm ischemia for purpose of test, which results in sufficient I/R injury to the kidney and perform nephrotomy, because dogs did not survive of 3 days after reperfusion for 90 minutes of warm ischemia in a pilot study (19). Although, many authors reported a peak damage of the renal function on 24 or 48 hours after reperfusion (9) and examined the subjects for 3 days after reperfusion (19), some authors investigated attenuation of renal I/R injury for 7 days (24). Thus the subjects were examined for 7 days after reperfusion. The aim of this study was to focus only on reperfusion injury after warm ischemia without the influence of other factors such as technical and manipulative differences during the nephrotomy. Renal function was evaluated by BUN and serum creatinine levels in this study. The levels of BUN and creatinine were significantly decreased in the treatment group 1 and 2 compare to control group at the 3rd, 5th and 7th day after reperfusion. And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion. 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 seemed to include a few well-described signal transduction pathways. These mechanisms include adenosine receptor mediated activation of adenosine triphosphate-gated potassium channels (4), nitric oxide synthesis (1), free radical generation (2) and the up-regulation of molecular chaperones (30).

Renal I/R injury results in decreased glomerular filtration and renal blood flow and increased urine output characterized by natriuresis and impaired concentrating ability. A number of drugs or chemicals have been used to prevent I/R injury in the

kidney. Namely, buckwheat, vitamin E, resveratrol and 21-aminosteroid were found to be effective in prevention of lipid peroxidation and general damage (18,29,33,35). Also, ascorbic acid (vitamin C) is transported into erythrocytes as dehydroascorbic acid, which is then reduced to ascorbate via a GSH-dependent reaction (6). As ascorbic acid represents the first line of antioxidant defense, is likely to be most vulnerable to oxidation. Thus, ascorbic acid may be sufficiently applied to protect vulnerable tissue from the oxidative stress. In this study, the levels of SOD, GSHPx and CAT were significantly increased in the treatment group 1 and 2 compare to control group at the 3rd, 5th and 7th day after reperfusion. And, there was significant difference between treatment group 1 and 2 at the 3rd day after reperfusion. This effect could be explained by a physical mechanism of free radicals washing. And the results was supported that ascorbic acid was a water-soluble antioxidant capable of scavenging free radicals and sparing other endogenous antioxidants from consumption (28).

Previous studies have shown that proinflammatory cytokines play a key role in I/R injury. I/R-induced renal TNF-alpha expression may result in renal cell injury by at least two distinct mechanisms: (i) direct cytotoxicity (induction of dysfunction and/or apoptosis) and (ii) neutrophil mediated tissue injury (25,27). In a recent study, I/R-induced renal TNF-alpha production has been found to be associated with impaired renal function and anti- TNF-alpha treatment results in diminished evident damage and improved renal function (13). Ascorbic acid can affect TNF-alpha production, since it reduces ROS. In the ischemic kidney sections, INF-gamma is localized in the shed proximal convoluted tubule cells.

This is considered to be the first study in which the procedure of premedication with ascorbic acid and irrigation-aspiration with heparinized saline was used to prevent the I/R injury in nephrotomy. Ascorbic acid is considered to be a scavenger of NOS. And also, irrigation-aspiration have a role of removal of emboli or scavenge nitric oxide species. It may have a physical mechanism of free radicals washing.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MEST) (No. 2010-0001356).

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개의 신장에 있어서 Ascorbic Acid에 의한 허혈/재관류 손상의 감소에 관한 연구

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요 약 : 본 연구의 목적은 개의 신장 절개술 모델에서 ascorbic acid를 전처치 하거나 관주 및 흡인시에 있어서, 허혈/재관류 손상의 감소와 신장 기능의 회복에 대한 효과를 평가하는데 있다. 성숙 잡종견 9두에서 신장절개술을 실시하였으며, hepa-saline 관주 및 흡인군와 ascorbic acid로 전처치 한 후 hepa-saline 관주 및 흡인한 군을 각각 실험군 1, 2로 놓았다. 신기능 검사와 항산화 효소 검사를 위해 신장절개술 후 0, 1, 3, 5, 7일에 혈액 샘플을 채취하였다. 그리고 7일 후 TNF-alpha, INF-gamma 검사를 위해 신장을 적출 보관하였다. 신장의 기능 검사에서 처치군 1과 2는 재관류 후 3, 5 그리고 7일째에 대조군과 비교하여 유의성 있게 감소 하였으며($p < 0.05$), 처치군 2는 재관류 후 3일째에서 처치군 1과 비교하여 유의성있게 감소하였다($p < 0.05$). 혈장에서의 항산화 효소의 활성치는 처치군 1과 2는 재관류 후 3, 5 그리고 7일째에 대조군과 비교하여 유의성 있게 증가 하였으며($p < 0.05$), 처치군 2는 재관류 후 3일째에서 처치군 1과 비교하여 유의성있게 증가하였다($p < 0.05$). TNF-alpha는 감소하고, INF-gamma는 증가하였다. 본 연구의 결과 신장의 관주 및 흡인의 과정은 신장의 관주 및 흡인의 과정은 신장의 허혈 및 재관류 손상을 감소 시키는 데에 효과가 있음을 시사하며, 또한, 외인성 ascorbic acid의 투여는 개의 신장 절개술에서 허혈 및 재관류의 손상을 감소시키며 신기능의 회복에 효과가 있음을 시사한다.

주요어 : 항산화제, 신장절개술, 개, 신장 허혈-재관류