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Attenuation of Renal Ischemia-Reperfusion Injury by Antioxidant Vitamins in Pigs

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Abstract : This study was to investigate the effects of ascorbic acid and alpha-tocopherol on the attenuation of renal ischemia-reperfusion (IR) injury in pigs. Ten pigs were subjected to 60 minutes of warm unilateral renal ischemia followed by removal of contralateral kidney and then divided into two groups. Treatment group was performed ascorbic acid and alpha-tocopherol pretreatment 2 days before operation and ascorbic acid with heparin-saline solution irrigation-aspiration. Otherwise, control group used only irrigation-aspiration of heparin-saline solution. Blood samples were collected from these pigs for measurement of serum blood urea nitrogen (BUN) and creatinine values, antioxidant superoxide dismutase (SOD) at pre, day 1, day 3, day 7 and day 14. The kidneys were taken for histopathologic evaluation after euthanasia on postoperative day 14. The levels of BUN were significantly increased in the control group on day 1, day 3 and day 7 ($P < 0.05$). And the level of creatinine was significantly increased in the control group on day 3 ($p < 0.05$). Activity of antioxidant enzymes in plasma revealed significant difference ($p < 0.05$) between control and treatment group at day 14. In histopathologic findings, treatment group was showed less damage than that of control group on the basis of renal tubular damage. It was concluded that ascorbic acid and alpha-tocopherol attenuated renal I/R injury in the pigs.

Key words **:** ascorbic acid, alpha-tocopherol, pig, renal ischemia-reperfusion, antioxidant.

Introduction

Tissue subjected to a period of ischemia undergoes morphological and functional damage, which increase during the reperfusion phase. Ischemia-reperfusion (IR) injury in the kidney is often observed in the renal operation (13). Thus, to decrease the degree of tissue damage is important to ameliorate cause of renal cell death, renal failure, delayed graft function, and renal graft rejection. 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). ROS also cause lipid peroxidation of cellular membranes and, hence, disruption of the structural integrity and capacity for cell transport and energy production, especially in the proximal tubule segment (1).

IR injury is thought to be related to a variety of circumstances such as harvest condition, donor age, cause of death and survival rate, prolonged warm and cold ischemia time, and surgical techniques of transplantation. The more severe the ischemia-reperfusion injury that occurs initially, the higher the incidence of rejection and graft dysfunction caused by significant regulation of major histocompatibility antigens ($P < 0.05$) (2,18). Therefore, reduction in primary IR injury would lead to better outcome for short- and long-term graft survival.

Like other organs, kidneys have enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic (tocoperols, carotenes, ubiquinol, ascorbic acid) antioxidant defenses to cope with this potential damage (12). The body contains an elaborate antioxidant defense system that depends on dietary intake of antioxidant vitamins and minerals, and the endogenous production of antioxidant compounds such as glutathione. Ascorbic acid and E and beta-carotene are the primary vitamin antioxidants. In addition to glutathione, there are numerous enzymes involved in the quenching or removal of free radicals (9).

A number of clinical studies showed that intravenous infusion of vitamin C or other antioxidants significantly reduces blood pressure in hypertensive patients (1,7).

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 (15). Such antioxidant, vitamin C has been used in diminution of free radical oxygen followed by IR injury of variety organs.

Many studies have shown that an inflammatory response induced by ischemia followed by reperfusion is largely

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responsible for tissue damage (6,20,22). IR injury leads to production of excessive amounts of ROS and reactive nitrogen species (RNS), causing oxidative stress which results in alternations in mitochondrial oxidative phosphorylation, depletion of adenosine triphosphate (ATP), an increase in intracellular calcium and activation of protein kinases, phosphatases, proteases, lipases and nucleases leading to loss of cellular function/integrity (18).

Increased production of ROS and subsequent elevated oxidative stress has been implicated in the development and progression of a wide range of conditions, including renal disease. There has been considerable interest in developing strategies to reduce oxidative stress, with many observational and animal studies supporting the use of antioxidant supplementation (16). The antioxidants in end-stage renal disease, was designed to test the effects of high-dogs (800 IU/day), oral, vitamin E supplementation-would support a role for antioxidant therapy (4,16).

Additionally, ascorbic acid improved renal hemodynamics and decreased oxidative stress, inflammation, and fibrosis in the porcine ischemic kidney (8).

The purpose of this study is to determine that antioxidant ascorbic acid and alpha-tocopherol therapy provide protection against IR injury in the same condition as the renal transplantation in pig kidney.

Materials and Methods

Ten pigs, 3-4 months old Landrace and Yorkshire mixed breeds of both genders weighing 36.5 ± 13.3 kg were provided from the agriculture livestock farm, Chungnam National University. The Laboratory Animal Care Advisory Committee of Chungnam National University approved the study protocol. These animals were acclimated and maintained on a standard diet for pigs, routine lighting cycle and room temperature, and demonstrated normal renal function before the surgical procedure.

The growing pigs were randomly assigned to the following one control group (n = 5), and treatment group (n = 5).

Control group (n = 5); the left kidney was freed from the perirenal tissue and fat. A bolus of 100 IU/kg of heparin was given intravenously at 4 minutes before ischemia. And then, left renal artery and vein were clamped with an atraumatic clamp. For inducing ischemia, hepa-saline [heparin (Choongwae Pharm., Korea, 1,000 IU) + Saline 500m*l*] was administrated through the renal artery (50 mmHg) and the fluid was suctioned from the renal vein during 60 minutes. After ischemia, the renal artery and vein were unclamped. And then, right nephrectomy was performed.

Treatment group (n = 5); Ascorbic acid [Vitamin C® Guju Pharm., Korea) 1,000 mg/head/day, IM] and alpha tocopherol [(Dong-A Pharm., Korea) 100 IU/head/day, PO] combination were given for 2 days before operation. The left kidney was freed from the perirenal tissue and fat. A bolus of 100 IU/kg of heparin was given intravenously in 4 minutes before ischemia. And then, left renal artery and vein were clamped with an atraumatic clamp. And vitamin C and hepa-saline (vitamin C 1,000 mg + heparin 1,000 IU + normal saline 500 m*l*) (32°C) were administrated through the renal artery (50 mmHg) and was the fluid was sucked from the renal vein during 60 minutes. After ischemia, the renal artery and vein were unclamped. And then, right nephrectomy was performed.

Surgical procedure

Pigs kept fasted for 24 hours prior to surgery, in order to prevent the possible adverse effects associated with anesthesia. The animals were premedicated with atropine sulfate (Atropine Sulfate®, Huons Co., LTD., 0.04 mg/kg, IM). After 10 minutes, Zoletil® (Tiletamine/Zolazepam, Virbac, France. 4.4 mg/kg, IM) was given for immobilization. Before tracheal intubation, thiopental sodium (Thionyl® Dai Han Pharm. Co., LTD., 15 mg/kg, IV) was administered intravenously and prophylactic antibiotic, ampicillin sodium (Penbrook®, Chong Kun Dang Co., 20 mg/kg, IV) and analgesic, meloxicam (Metacam®, Boehringer Ingelheim Co., 0.2 mg/kg, IM) therapy was given.

The anesthesia was induced and maintained with isoflurane 2% and 100% pure oxygen supply. Laparatomy was performed by midline incision. The left kidney was isolated, and then both the renal artery and vein were clamped. After 60 minutes of warm ischemia, the vessels were unclamped, and the right kidney was removed. And then irrigation-aspiration was performed in both groups.

During the operation, the pigs were given intravenous fluid (Hartmann sol, 5 m*l*/*kg*/h). Postoperatively, the pigs were allowed free access to water and food after 24 hours of surgery.

Renal function

Serum samples were collected by venipuncture from the left juglar vein at each point and each sample was kept in tube. After each sample was coagulated, using centrifugation with 650 *g* for 15 minutes, the serum was segregated. Blood urea nitrogen (BUN) and creatinine levels were measured from serum samples taken on day 0 (pre-operative), day 1, day 3, day 7 and day 14, from jugular vein, using a blood chemistry analyzer (IDEXX Vetest 8008, USA). The results are expressed as mg/d*l*.

Antioxidant enzyme activity in plasma

Blood samples were collected using EDTA as anticoagulant, and centrifuged at 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 on ice until assaying or freeze at –80°C Samples were stored at –80°C in deep freezer. Superoxide dismutase (SOD) was measured with optical density (OD) assay kit-WST (Dojindo, USA) and Benchmark microplate reader (Bio-rad, USA). SOD activity was assessed by measuring the dismutation of SRs generated by XO and hypoxanthine in a labware 96 well format. The activity was recorded spectrophotometrically at 450 nm. The enzyme activity was calculated as U/m*l*.

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

SOD, which exists in mitochondrial (Mn-SOD) and cytoplasmic forms (Cu/Zn-SOD), catalyses the conversion of two O_2•– molecules into H_2O_2 and O_2. Because H_2O_2 is a potential source for OH• radicals, two additional protective enzymes: catalase (CAT) and glutathione peroxidase (GPx) transform it (16).

Histopathologic examination

The left kidney of normal, control and treatment group were taken for histopathologic examination after euthanasia on postoperative day 14. The kidneys were fixed in 10% neutral buffered formalin. Samples were dehydrated in 70% to 100% alcohol, and xylene was used for clearing samples. Tissue were embedded in paraffin, sectioned (5 μm thick), stained with H-E, and examined with light microscope.

Statistical analysis

All values are expressed as means ± SD of determinations for all pigs in the group. Data were analyzed using analysis of variance followed by two-way repeated measures analysis (ANOVA) followed by Student *t*-test and a *p* value below 0.05 or 0.01 was considered statistically significant.

Results

Renal function

Serum creatinine levels, measured as an index of kidney function, were nearly normalized to 1.39 ± 0.26 mg/d*l*, 1.32 ± 0.12 mg/d*l* in the control and treatment group prior to euthanasia after 14 days of reperfusion (normal range, 0.50 – 2.10) (Fig 1). Mean serum creatinine level (in milligrams per deciliter) in treatment group was significantly lower than in control group on day 3 (4.46 ± 1.27 vs. 2.53 ± 0.93) ($P < 0.05$).

It is also measured BUN as a second index of the kidney function in this treatment group. Similar to serum creatinine, the levels of BUN in the control group and treatment group on pre (day 0) were 9.9 ± 4.85 mg/d*l* and 10.48 ± 5.72 mg/d*l* (normal range, 10.0 – 12.0 mg/d*l*) respectively.

Serum BUN levels were nearly normalized to 20.80 ± 11.98 mg/d*l* and 19.3 ± 6.28 mg/d*l* in the control and treatment group prior to euthanasia after 14 days of reperfusion (Fig 2). Mean serum BUN levels (in milligrams per deciliter) in treatment group were significantly lower than in control group on day 1 (134.83 ± 11.67 vs. 53.00 ± 17.50), day 3 (81.87 ± 25.17 vs. 35.00 ± 23.40), and day 7 (37.77 ± 16.48 vs. 19.43 ± 4.11), respectively ($P < 0.05$).

Antioxidant enzyme activity in plasma

The activities of SOD enzyme in plasma exposed to 60 minutes of ischemia were observed at pre (day 0) and day 1, day 3, day 7 and day 14 after reperfusion are shown as follows.

The specific activities of SOD in control group exposed to 60 minutes of ischemia insult were observed for 14 days after reperfusion. The levels of SOD activity were 22.85 ± 1.69 U/m*l* (Control) and 21.86 ± 2.37 U/m*l* (Treatment) followed before surgical operation (Fig 3). There was significant difference ($P < 0.05$) between treatment group and control

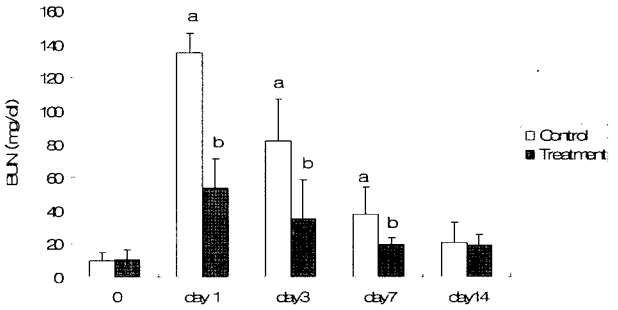

Fig 2. Serum BUN levels in pigs after 60 minutes of ischemia-reperfusion. The values are expressed as mean ± SD (n = 5).
a,b: Values marked with different letters (a, b) represent significant different means ($p < 0.05$) at same time point.

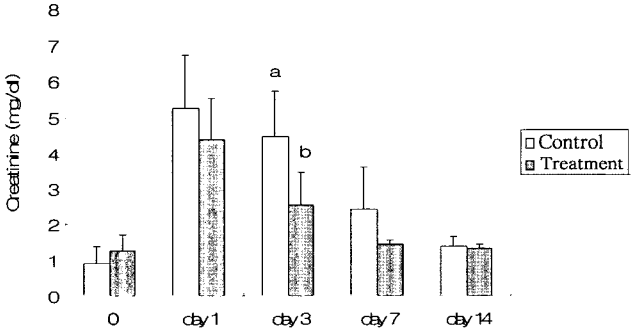

Fig 1. Serum creatinine levels in pigs after 60 minutes of ischemia-reperfusion. The values are expressed as mean ± SD for all groups (n = 5).
a, b: Values marked with different letters (a, b) represent significant different means ($p < 0.05$) at same time point.

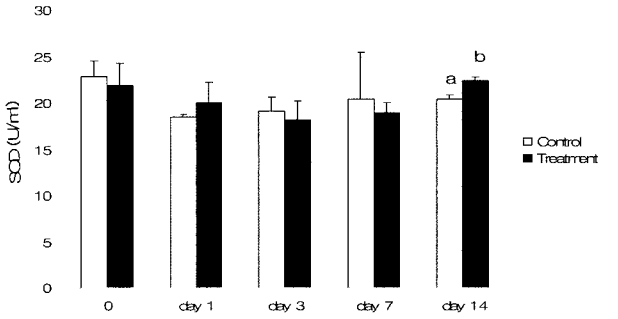

Fig 3. The activity of total superoxide dismutase (SOD) in plasma was observed at pretreatment and day 1, day 3, day 7 and day 14 after reperfusion. The values are expressed as mean ± SD for all groups (n = 5).
a,b: Values marked with different letters (a, b) represent significant different means ($p < 0.05$) at same time point.

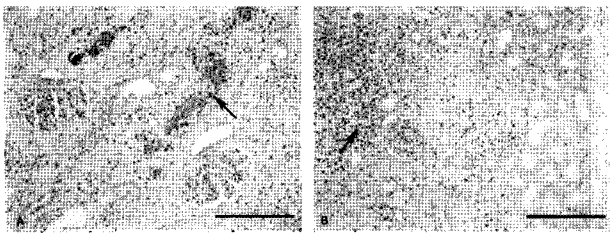

Fig 4. Kidney from the control group. Note severe and moderate necrosis of tubular epithelium, calcification of the necrotic tubules (A, arrow), and infiltration of the inflammatory cells (B, arrow). (H-E stain Bar A = 10 μm, B = 100 μm)

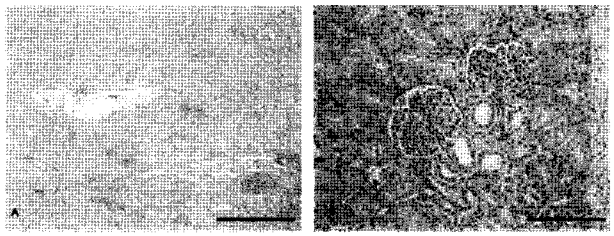

Fig 5. Kidney from the treatment group. Note moderate and mild necrosis of the tubular epithelium, and regenerating tubules. (H-E stain Bar A = 10 μm, B = 100 μm).

group at the day 14 after reperfusion.

Histopathologic findings

At the autopsy 14 days after the operation, kidney from the control group administered to heparin-saline irrigation-aspiration observed severe and moderate necrosis of the tubular epithelium, infiltration of the inflammatory cell, interstitial fibrosis, and mineralization (Fig 4).

In contrast, kidney of the treatment group administered to heparin-saline and ascorbic acid and alpha-tocopherol pretreatment therapy before 2 days of surgery were found moderate and mild necrosis and regeneration of the tubular epithelium and infiltration of the inflammatory cells (Fig 5).

Discussion

Renal IR injury results in decreased glomerular filtration and renal blood flow (RBF), and increased urine output characterized by natriuresis and impaired concentrating ability. A number of drugs or chemicals have been used to prevent IR 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 (11,17,21,23). Also, ascorbic acid is transported into erythrocytes as dehydroascorbic acid, which is then reduced to ascorbate via a reduced glutathione (GSH)-dependent reaction (11). 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.

Renal function was evaluated by BUN and serum creatinine levels in this study. The levels of BUN and creatinine were significantly increased in the control group on day 1, day 3 and day 7 ($P < 0.05$). And the level of creatinine was significantly increased in the control group on day 3 ($p < 0.05$). But antioxidant enzyme activity (SOD) was not significantly different between two groups and slightly significant difference in day 14 of operation ($P < 0.05$). This effect could be explained both by a physical mechanism of free radicals washing. The results supported that ascorbic acid was a water-soluble antioxidant capable of scavenging free radicals and sparing other endogenous antioxidants from consumption (3,10).

In histopathologic and morphologic findings, control group revealed severe necrosis of tubular system, while in treated group, damage of the tubule revealed less than that of control group. Previous studies have demonstrated that ischemia irreversibly damages the distal segments of the proximal tubules (S_3) whereas more proximal segments suffer reversible injury after a short period of normothermic ischemia (5,19).

Although it did not show differences of segment of tubule in this study, the S_3 segment might undergone necrosis and shed into lumen of the tubule which was considered to be the basis for the decrease in the glomerular filtration rate which is indicated by a significant increase in the renal function levels (serum BUN and creatinine).

This is the first study in which the procedure of antioxidant pretreatment therapy accompanied by irrigation-aspiration was used to prevent the IR injury in the kidney. Although, it is not known yet whether irrigation-aspiration has a role of removal of emboli or scavenge nitric oxide species, it may have a physical mechanism of free radicals washing.

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돼지의 신장에서 Antioxidant Vitamins에 의한 허혈 및 재관류 손상의 감소에 관한 연구

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요 약 : 돼지에서 신장의 허혈-재관류 손상의 감소에 대한 비타민 C와 E의 영향을 알아보기 위하여 본 연구를 실시하였다. 돼지 10두를 2개의 군으로 구분하여, 60분 동안 체온과 같은 허혈을 한쪽 신장에 유발하고 반대쪽 신장은 절제하였다. 처치군은 수술 2일전 비타민 C와 E를 이틀 동안 전처치하고, 그 뒤에 수술 중 비타민 C와 heparin이 첨가된 생리식염수를 관주-흡인 하였다. 대조군은 heparin이 첨가된 생리식염수의 관주-흡인만을 하였다. Blood urea nitrogen, creatinine 및 antioxidant superoxide dismutase (SOD)를 측정하기 위하여 수술 전, 수술 후 1, 3, 7, 14일에 혈액 샘플을 채취하였다. 수술 후 14일에 안락사를 시키고, 병리조직검사를 위하여 신장을 적출하였다. BUN은 대조군과 처치군 사이에 수술 후 1일, 3일 또는 7일에 유의성이 있는 차이가 인정되었다 ($p < 0.05$). Creatinine은 대조군과 처치군 사이에 수술 후 3일에 유의성이 있는 차이가 인정되었다 ($p < 0.05$). 혈장에서의 항산화 효소의 활성은 대조군과 처치군 사이에서 수술 후 14일에 유의성이 있는 차이가 인정되었다 ($p < 0.05$). 병리조직 검사 결과에서 처치군이 대조군 보다 더 적은 신장 세뇨관에서의 손상의 정도를 보였다. 비타민 C와 E는 돼지에서 신장의 허혈-재관류 손상을 감소시켰다.

주요어 : 비타민 C, 비타민 E, 돼지, 신장 허혈-재관류, 항산화제