Effect of Cell Permeable Peptide of c-Jun NH2-Terminal Kinase Inhibitor on the Attenuation of Renal Ischemia-Reperfusion Injury in Pigs

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Effect of cell permeable peptide of JNK inhibitor on the attenuation of renal ischemia/reperfusion injury in pigs

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Authorship and conflict of interest statement

I declare that I have no conflict of interest in connection with this paper.

Abstract

The outcome of organ transplantation has been improved by better immunosuppressive drugs, surgical techniques and management of systemic conditions. However, ischemia/reperfusion injury remains one of the challenges that affect graft survival.

In this study, we used a new technique employing protein transduction domain (PTD) to investigate whether the inhibition of c-Jun NH_2-terminal kinase (JNK) pathway attenuates renal ischemia/reperfusion injury. In a porcine renal ischemia/reperfusion model, a PTD-JNK-inhibitor (JNKI) was administered into renal artery. And a PTD-JNKI was then taken into the various cells containing the vascular endothelial cells. The uptake of these was conducted by endocytosis using PTD. Serum creatinine and blood urea nitrogen concentrations were lower in the PTD-JNKI group than in the control group. In addition, renal tissue blood flow was well maintained in PTD-JNKI group, resulting in a lower level of tissue injury and fewer apoptotic cells. These results suggest that the PTD technique improves renal transplantation outcomes.

Introduction

The number of cadaveric donors remains insufficient compared with the increasing number of transplant candidates[1]. Since the number of cadaveric donors is very small especially

in Japan, kidneys occasionally need to be extirpated from marginal donors. In these cases, acute tubular necrosis resulting in delayed graft function or primary non function is the most common complication[2].

The short term inflammatory response induced by ischemia/reperfusion injury is characterized by induction of a proinflammatory cytokine cascade, expression of adhesion molecules and cellular infiltration[3]. Interleukin-1β and tumor necrosis factor-α (TNF-α) are well known proinflammatory cytokines. c-Jun NH_2-terminal kinase (JNK), a stress activated protein kinase, forms a subgroup of the mitogen-activated protein kinase (MAPK) superfamily[4]. The JNK pathway is closely related to apoptosis in ischemia/reperfusion injury[5]. In addition, JNK activation is mediated by reactive oxygen species in TNF-α-induced apoptosis[6].

The authors previously reported attenuation of ischemia/reperfusion injury by the use of FR167653 or gabexate mesilate in large animal models[7-9]. In addition, a new technique of efficient substance induction into cells using protein transduction domain (PTD) has recently been reported[10]. PTD has many amino acids with a positive electric charge, so it adheres strongly to the negatively charged cell membrane lipid bilayer. Thus PTD can transduce a wide variety of cargo into cells, from small intermediate molecules to liposomes[11].

We think this model is not pure warm or cold ischemia experiment, but it is similar to clinical situation. Extirpation from marginal donors needs strict management from early stage.

When a donor faces death and kidney bloodstream is insufficient, kidneys of a donor are state of warm ischemia. We think administering a protective drug to a donor from this point in time is needed. Thus we have to administer a drug at room temperature. That is why this model is not pure warm or cold ischemia.

In this study, we evaluated the effect of cell permeable peptide of a JNK inhibitor (JNKI) to attenuate renal ischemia/reperfusion injury in a porcine model, and explore the possibilities for adapting the technique to human donors.

Materials and Methods

This study was reviewed by the Committee of Ethics on Animal Experiments at Kyushu University and conducted according to the Guidelines for Animal Experiments of the Graduate School of Medical Sciences, Kyushu University, Law No. 105, and Notification No. 6 of the Japanese Government.

Operative Procedures

Twelve female hybrid Landrace swine weighing 20 to 30 kg were used. General anesthesia was induced with intramuscular midazolam (10 mg/pig), butorphanol tartrate (1 mg/pig) and medetomidine hydrochloride (1 mg/pig) followed by intravenous pentobarbital (25 mg/kg) and pancuronium bromide (0.1 mg/kg). Pancuronium bromide was supplemented as

needed. The animals were then intubated and ventilated mechanically with oxygen (1 L/min) and 1 to 2% of sevoflurane with a tidal volume of 20 mL/kg at 12 cycles/minute. A central venous line was placed into the right external jugular vein with a cutdown technique, and physiological saline was infused during the operation at 300 ml/hr. The pulse rate and oxygen saturation were continuously monitored. Cefalotin sodium (1 g/pig) was administered intravenously 30 minutes before the start of the operation.

Laparotomy was performed by ventral midline incision. The left kidney was isolated, and then both the renal artery and vein were encircled with tapes. The left ureter was isolated and encircled similarly. The right kidney was removed. The left renal artery and vein were clamped. The renal vein was clamped proximal to a lumbar vein. The ureter was also clamped to prevent reflux of blood into the kidney through the vascular network around the renal hilum. The left kidney was perfused with physiological saline at room temperature from the renal artery until discoloration was observed. The perfusate was drained from a lumbar vein. After the lumbar vein was ligated, 10 ml of the PTD-JNKI solution (experiment) or physiological saline (control) was infused into the renal artery at room temperature. The kidney was placed under the small intestine to be kept at body temperature for 90 minutes.

After 90 minutes of ischemia, all the vessels and the ureter were unclamped. After observation for 60 minutes, the left kidney was covered and secured by the retroperitoneum and

the abdomen was closed. Postoperatively, the endotracheal tube was removed and the animals were allowed access to water and food ad libitum. On postoperative day five, tissue samples were obtained from the left kidney and the animals were euthanized by overdose of pentobarbital.

Experimental Groups

PTD-JNKI was purchased from Sigma Aldrich (Ishikari, Japan). The animals were assigned randomly to one of two groups. The investigators were blind with regard to the groups. The PTD-JNKI group (n = six) received PTD-JNKI solution (10 μM). The control group (n= six) received physiological saline.

Peptide Synthesis

PTD-JNKI peptide synthesis was requested from Sigma Ardrich. 11 arginine connected peptides were used for PTD. JNKI was selected as a material for preventing activation of the c-Jun NH_2-terminal kinase pathway. It was hypothesized that it would attenuate ischemia/reperfusion injury and apoptosis. PTD-JNKI peptide sequence was RRRRRRRRRRRGGRPKRPTTLNLFPQVPRSQDT. Molecular weight of this peptide was 4542.16. We conjugated fluorescein to the N terminal of the peptide. This peptide was purified to >80% purity.

Confirmation of Induction of PTD-JNKI into the Cells

To confirm the induction of PTD-JNKI into the cells, we used fluorescein-conjugated PTD-JNKI peptide and fluorescence microscopy.

Biochemical Parameters

Serial blood samples were collected before the operation and 1, 6, 24, 48, 72, 96 and 120 hours after reperfusion. The samples were centrifuged, and the serum was stored at -80°C until analysis. Serum creatinine and blood urea nitrogen (BUN) concentrations were determined by enzymatic assay (SRL model 7170 autoanalyzer for creatinine and model 7450 autoanalyzer for BUN; Hitachi, Tokyo, Japan).

TNF-α Measurement

Blood samples were collected before operation and one hour after reperfusion. The samples were centrifuged, and the serum was stored at -80°C until analysis. In these samples, TNF-α concentrations were determined by using a commercial porcine TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Porcine TNFα, Pierce Endogen, Inc., Rockford, IL, U.S.A.). Absorbency of ELISA plates was measured at 450 nm by the use of a spectrophotometer (Immuno Mini NJ-2300, Nalge Nunc Int., New York, NY, U.S.A.).

Evaluation of Renal Blood Flow and Vascular Resistance

Renal tissue blood flow was measured with a laser Doppler flow meter (Advance ALF21R; Unique Medical, Tokyo, Japan) on the cranial, middle, and caudal portions of the

kidney before clamping, during ischemia, at 15, 30, 45, 60 minutes and at 120 hours after reperfusion. Doppler flow study was performed at the three different points of the kidney before clamping, at 15, 30, 45, 60 minutes and at 120 hours after reperfusion with a Doppler flow meter (SSD-5500; ALOKA Prosound, Tokyo, Japan). Resistive index (RI) and pulsatility index (PI) were calculated at each point on the interlobular arteries according to the following formula: $RI = (V_{max} - V_{min})/V_{max}$, $PI = (V_{max} - V_{min})/V_{mean}$, where V_{max} denotes maximum velocity, V_{min} denotes minimum velocity, and V_{mean} denotes mean velocity.

Histological Examination.

All the animals were sacrificed and the left kidney of each retrieved on post-operative day five. The tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned in 4 μm slices and mounted on slides. After deparaffinization, each specimen was stained with hematoxylin and eosin to assess the level of histological tissue injury. The slides were evaluated in terms of dilatation of proximal tubules, eosinophilic casts in distal tubules, loss of brush borders, detachment of tubular cells, interstitial edema, whole tubular necrosis, neutrophil infiltration, and interstitial hemorrhage. The findings were graded 0-3 (0; < 5% injury per 10 high-power fields (HPFs); 1: 5-24% injury per 10 HPFs; 2:25-49% injury per 10 HPFs; 3: >50% injury per 10 HPFs. The samples were randomized and blindly examined by light microscopy independently by two investigators capable of pathologic interpretation.

Apoptotic Index Using Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick End-Labeling (TUNEL) Stain

For the detection of DNA breaks, the TUNEL stain (In Situ Apoptosis Detection Kit, TaKaRa, Otsu, Japan) was used. After pretreatment with proteinase K for 15 minutes at room temperature, endogenous peroxidase activity was blocked with 3% H_2O_2 for five minutes, also at room temperature. Equilibration buffer was applied to the sections for 10 seconds at room temperature and they were then incubated with working strength terminal deoxynucleotidyl transferase (TdT) enzyme for 60 minutes at 37°C in a humidification chamber. The sections were then incubated with anti-digoxigenin conjugate for 30 minutes at room temperature in a humidification chamber. Peroxidase substrate was applied for three to six minutes at room temperature. The sections were counterstained with 0.5% methyl green for 10 minutes and examined by light microscopy.

The apoptotic index (AI) was defined as the ratio of TUNEL positive cells to 1000 renal tubular epithelial cells in a clearly labeled area at ×400 magnification. Serial methyl green stained sections were also analyzed to avoid misinterpretation of necrotic cells.

Statistical Analysis

All results are presented as mean ± standard deviation. Differences in serum creatinine (sCr), serum blood urea nitrogen (sBUN), tissue blood flow, TNF-α expression and vascular

resistance over time were evaluated with repeated-measures analysis of variance (ANOVA) with a post hoc test. Differences in the grade of the histological injury were evaluated with a Mann-Whitney test. A p-value < 0.05 was considered statistically significant.

Results

Operative Findings and Postoperative Course

After reperfusion, the left kidney of all subjects became firm and the color returned to normal. All animals in both groups walked and ate from starting on post-operative day 1. All animals in both groups survived until they were sacrificed on the fifth day. No signs of intra-abdominal infection were noted. The color of the left kidney was normal and a normal amount of urine was present in the bladder in all subjects.

PTD-JNKI Transduction

PTD-JNKI peptide was successfully induced into the cells via infusion of the renal artery at room temperature. The fluorescein-labeled PTD-JNKI peptide was visualized in the vascular endothelial cells 30 minutes after infusion (Figure 1).

Renal function

In both groups, serum BUN concentrations began to increase starting six hours after ischemia/reperfusion, and reached a peak on post-operative day 2 (Figure 2A). However, BUN

concentrations were lower in the PTD-JNKI group than in the control group during the entire post-operative period ($P<.0001$). The change in serum creatinine concentrations was similar to that of BUN concentrations (Figure 2B). Serum creatinine concentrations were also lower in the PTD-JNKI group than in the control group ($P<.0001$).

Renal Blood Flow

In both groups, renal tissue blood flow, as determined by Doppler flow meter, was decreased between clamping and unclamping, and then began to increase gradually (Figure 3A). On post-operative day five, renal tissue blood flow exceeded the pre-ischemic level. There was no statistically significant difference between groups in renal tissue blood flow ($P=0.8461$).

Vascular Resistance (Pulsatility Index)

In the control group, PI decreased immediately after reperfusion and reached minimum values in 30 minutes (Figure 3B). PI then increased and reached maximum values on post-operative day five. In the PTD-JNKI group, PI also decreased immediately after reperfusion, but maintained a steady value from 15 minutes until 60 minutes of ischemia (Figure 3B). The PI increased on post-operative day five and reached the pre-ischemic level. The PI in the PTD-JNKI group was lower than in the control group ($P<0.05$).

TNF-α Measurement

In both groups, the levels of serum TNF-α one hour after ischemia/reperfusion were

higher than that before ischemia/reperfusion. This difference was not statistically significant (P=0.1929).

Histological Examination

On post-operative day five, renal tissue samples from the control group demonstrated severe dilatation and loss of brush borders in proximal tubules, eosinophilic casts in distal tubules, mild interstitial edema, neutrophil infiltration and interstitial hemorrhage. The severity of injury was attenuated in all of these parameters in the PTD-JNKI group (Injury score: 10.8±3.13 vs 6.33±2.25; P=.03).

Apoptotic Index Using TUNEL Stain

On post-operative day five, fewer positively stained cells were identified in the PTD-JNKI group than in the control group. Thus, AI was significantly reduced in the PTD-JNKI group (AI: 58.3±13.5 vs 12.8±4.75; P=.0039).

Discussion

The present study revealed that PTD-JNKI was induced into renal endothelial cells by perfusion from the renal artery in the porcine model. Furthermore, PTD-JNKI induced into renal cells attenuated ischemia/reperfusion injury and reduced apoptotic tubule cells. As a result, this study suggests that induction of PTD-JNKI into renal endothelial cells may improve renal

transplantation results from marginal donors.

The authors established the renal ischemia/reperfusion injury model in dogs in previous reports[7,9]. Similar techniques were used for the present porcine model to confirm whether PTD-JNKI attenuates renal ischemia/reperfusion injury, and whether it may consequently improve outcomes of renal transplantation. Because serum BUN and creatinine levels were slightly elevated when kidneys were exposed to a 60-minute warm ischemia in a pilot study, it was decided to expose the kidneys to a 90-minute warm ischemia. In addition, the contralateral kidney was removed in order to simplify the model.

JNK phosphorylates are not only regulatory sites in the N-terminus of the transcription factor c-Jun but also in other transcription factors such as Elk-1 and p53. Cell apoptosis is induced by the activation of JNK[5]. JNK activation is mediated by reactive oxygen species in tumor necrosis factor-α induced apoptosis[6]. Many results have been reported that the inactivation of JNK pathways attenuates ischemia/reperfusion injury in heart[12,13], brain[14,15], islets[16-18], liver[19] and kidney[20]. Thus, a JNK inhibitor was chosen in the present study to attempt to attenuate renal ischemia/reperfusion injury.

Previously, intracellular transport of materials was thought to be limited to those of small size and molecular weight. Recently, however, some articles have reported that large molecular weight materials can be effectively introduced intracellularly using a mechanism

called PTD[10,11]. PTD was first reported in 1988 as a part of TAT protein of human immunodeficiency virus[21]. The Drosophila melanogaster homeobox protein Antennapedia[22,23] and herpes simplex virus protein VP22[24] are also well known examples of PTD. These PTDs have many amino acids, such as arginine and lysine, with a positive electric charge. The mechanism of introduction of PTD into cells was previously unclear. However, some reports have recently described that the mechanism of induction is due to endocytosis. Positively charged PTD adheres to the negatively charged cell membrane lipid bilayer strongly[25]. Subsequent transport is performed by endosomes[26,27].

In the present study, a peptide consisting of 11 arginine was used as a PTD because this PTD was reported to be more efficient for transduction into cells[28]. Induction into renal endothelial cells using this PTD was confirmed by fluorescence microscopy. PTD-JNKI was induced into cells at room temperature, in order to replicate the clinical environment. Further examination in varying environments is necessary.

Renal transplantation is the only curative treatment for end stage renal failure. However, donor shortage remains a major problem in transplantation of many organs[29,30]. Therefore, kidneys must sometimes be used from marginal donors, such as aged organs or those with prolonged ischemic time. Ischemia/reperfusion injury is one of the factors affecting the outcomes of renal transplantation, especially from non-heart-beating donors[2]. This type of

injury needs to be attenuated to improve the outcomes of transplantation from such donors. In the authors' previous communications, they reported the effects of a cytokine-suppressive agent FR167653 or a synthetic protease inhibitor gabexate mesilate to attenuate ischemia/reperfusion injury on the outcomes of renal or pancreatic transplantation in animal models[7-9].

In ischemia/reperfusion injury, disturbance of micro-circulation is largely due to endothelial damage, leading to an increase in vascular permeability[31]. It then causes leukocyte plugging, vasoconstriction, and hemoconcentration[32,33]. In short, leukocytes localize and adhere to adhesion receptors of the endothelium, such as intercellular adhesion molecule-1 (ICAM-1), resulting in immobilization and diapedesis of leukocytes. ICAM-1 expression is then increased by IL-1 and TNF-α[34]. In the present study, renal tissue blood flow decreased during ischemic time, gradually increased after unclamping, and exceeded the pre-ischemic level on post-operative day five. Although there was no significant difference in renal tissue blood flow between groups, PI, reflecting peripheral vascular resistance, was lower in the PTD-JNKI group than in the control group throughout the experimental period especially during the renal injury period. The reason why tissue blood flow did not increase in the PTD-JNKI group despite the low peripheral vascular resistance remains unknown. The decrease in the vascular resistance may have increased tissue oxygen supply, resulting in the improvement of BUN and creatinine concentrations.

ICAM-1 is intimately involved in acute renal failure caused by ischemia/reperfusion injury. TNF-α accelerates the expression of ICAM-1. As serum TNF-α was reported to increase one hour after ischemia/reperfusion in an animal model[35], we examined the concentrations of serum TNF-α one hour after ischemia/reperfusion. In this study, there was no significant difference in the serum TNF-α concentration between the control and PTD-JNKI groups. Measuring TNF-α at a later point or in the tissue samples instead of serum may have produced a different result.

In clinical renal transplantation, characteristic histological findings in acute tubular necrosis are dilatation of the proximal tubules, degeneration of the tubular epithelium, interstitial edema, cellular infiltration, and casts in the distal tubules[36]. The authors previously reported that loss of brush borders reflected acute tubular necrosis well[9]. In the present study, several additional parameters were evaluated (loss of brush borders, detachment of tubular cells, whole tubular necrosis, interstitial hemorrhage). Using these parameters, the extent of injury in the control group was greater than in the PTD-JNKI group. The most noticeable differences observed were dilatation of the proximal tubules, loss of brush borders in the proximal tubules, and eosinophilic casts in the distal tubules.

The control group also contained many more apoptotic tubular cells as compared to the PTD-JNKI group. Apoptosis is the principal mechanism leading to organ damage in renal

ischemia/reperfusion injury[37].

In conclusion, induction of a cell permeable JNK inhibitor peptide attenuates renal ischemia/reperfusion injury in pigs. This method may improve the results of renal transplantation in humans and expand donor availability.

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Figures

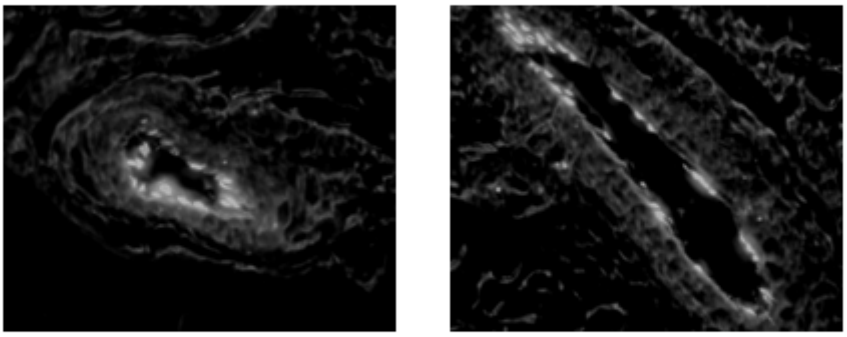

Figure 1. Induction of PTD-JNKI into renal endothelial cells was observed by fluorescence microscopy. A. Arterial endothelial cells. B. Venous endothelial cells (Original magnification ×400).

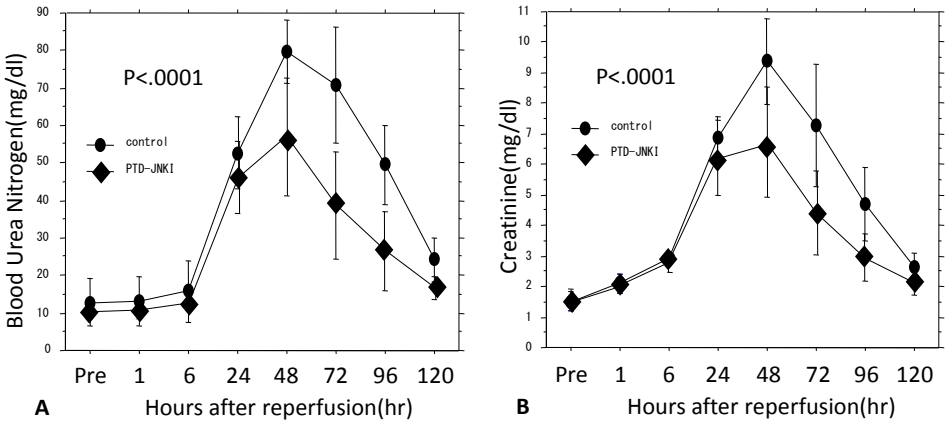

Figure 2. Renal function. A. Serum BUN concentrations were lower in the PTD-JNKI group. B. Serum creatinine concentrations were lower in the PTD-JNKI group. Values are mean±SD; n = six animals per group.

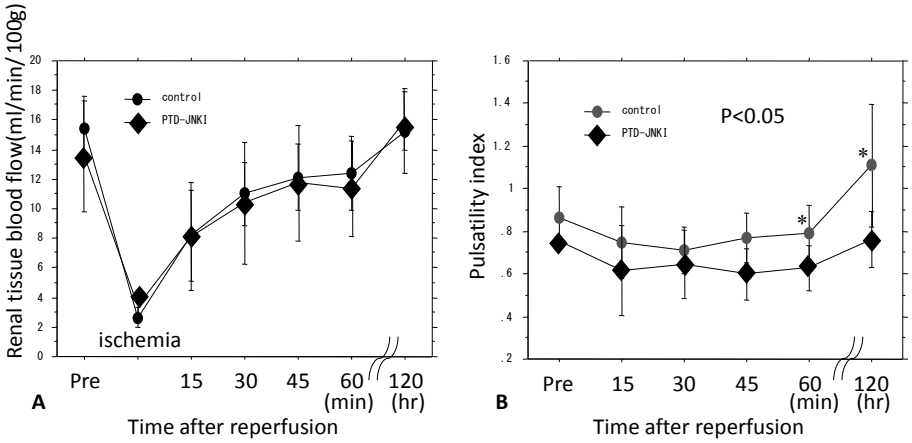

Figure 3. Renal blood flow. A. Renal tissue blood flow (RTBF) measured with a Doppler flow-meter. There is no significant difference between the control group and PTD-JNKI group (P=0.8461). B. Pulsatility index (PI) measured by echo Doppler. PI was lower in the PTD-JNKI group than in the controls throughout the study period. * P<0.05 versus control group by one-way ANOVA. Values are mean±SD; n = six animals per group.

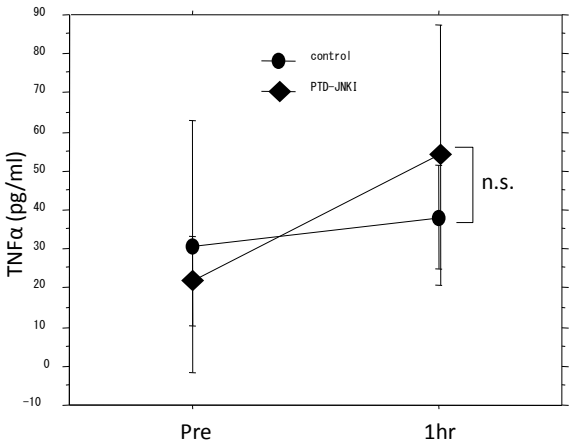

Figure 4. Serum TNF-α concentrations before and one hour after ischemia/reperfusion. The concentrations tended to increase, but there is no difference between the control and PTD-JNKI group. Values are mean±SD; n = six animals per group.

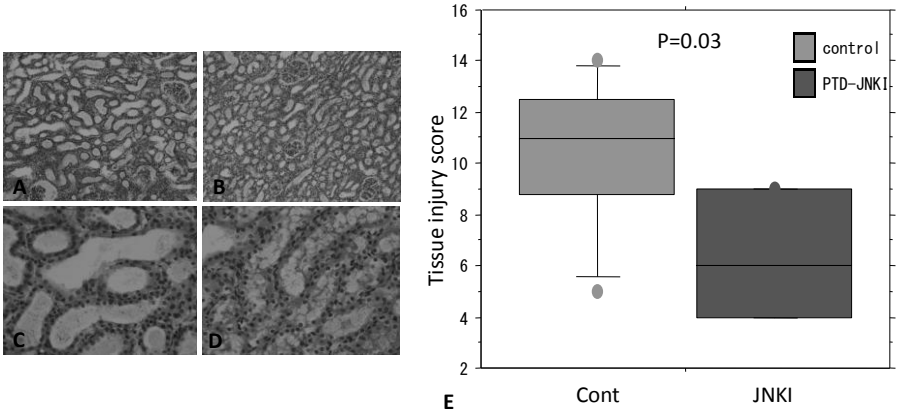

Figure 5. Representative microscopic findings at postoperative day five. A&C. Control group: severe dilatation of the proximal tubules and loss of brush borders, mild interstitial edema, neutrophil infiltration and interstitial hemorrhage. B&D. PTD-JNKI group: severity of injury was attenuated in all parameters. (A&B. Original magnification×100; C&D. Original magnification×400) E. Injury score: 10.8±3.13 vs 6.33±2.25; P=.03 Values are mean±SD; n = six animals per group.

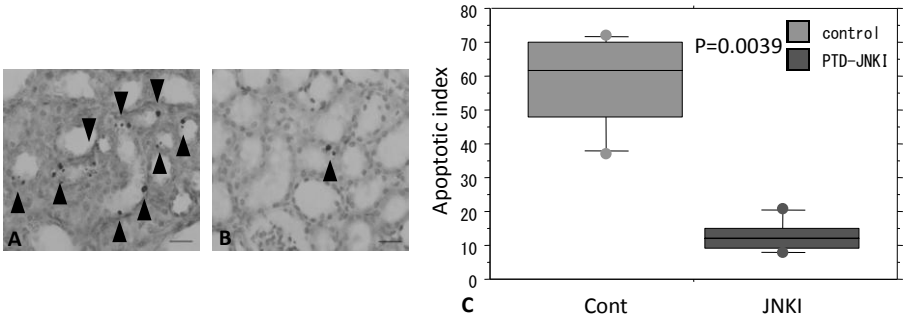

Figure 6. TUNEL stain at postoperative day five. A. In the control group, many apoptotic cells were confirmed in renal tubule cells (arrow). B. In the PTD-JNKI group, the number of apoptotic cells was remarkably decreased (Original magnification×400). C. Apoptotic index (AI). AI was the ratio of TUNEL positive cells to 1000 renal tubular epithelial cells in a clearly labeled area at ×400 magnification. (AI: 58.3±13.5 vs 12.8±4.75; P=.0039) Values are mean±SD; n = six animals per group.