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Full Length Research Paper

Effects of penehyclidine hydrochloride and anisodamine on acute kidney injury induced by two-hit Rats

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Anticholinergics have effects on organs with hemorrhage-reperfusion injury. The present study investigated the benefit effects of penehyclidine hydrochloride (PHC) and anisodamine (ANI) on hemorrhage-reperfusion and lipopolysaccharide (LPS) (two hits) induced acute kidney injury (AKI). Administration of PHC and ANI do not only remarkedly reduced the plasma concentrations of tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), but also reduced malondialdehyde (MDA) content, myeloperoxidase (MPO) activity, and enhanced superoxide dismutase (SOD) activity. Histopathological observation showed that PHC and ANI treatment markedly relieved renal histopathological damages, and inflammatory cells infiltration. Furthermore, immunohistochemistrical study presented that intercellular adhesion molecule-1 (ICAM-1) was increased after two hits management, which could be attenuated by PHC and ANI treatment. These data indicated that PHC and ANI had the benefit effects on AKI resulting from two-hits in rats. The effects of PHC treatment were better than ANI.

Key words: Anisodamine, penehyclidine hydrochloride, two hits, acute kidney injury.

INTRODUCTION

It is generally accepted that severe infection, trauma, and hemorrhagic shock can activate inflammatory action leading to systemic inflammatory response syndrome (SIRS). In recent years, it has been increasingly recognized that SIRS could contribute to the induction of acute kidney injury (AKI) (Zager et al., 2005). In the surgical intensive care unit, critically sick patients suffered from various insults that resulted in their organ function deterioration, while inflammation after renal ischemia-reperfusion injury (IRI) was a leading contributor to renal cell death, and SIRS was an important factor and the potential mechanism to AKI (Lee et al., 2007; Aregger et al., 2009).

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The two-hit theory has become increasingly accepted. It is believed that a less severe traumatic or hemorrhagic insult as “the first hit” to activate an inflammatory environment; and the subsequent insults, infectious or non-infectious, as “the second hit” may amplify the pre-existing inflammatory state into SIRS (Saadia and Schein, 1999). In order to investigate the clinical patho-physiology changes of AKI which resulted from various insults, the two-hit model was duplicated by two-hits of hemorrhage-reperfusion and lipopolysaccharide (LPS), which can reflect the pathology, pathophysiology and complexity of clinically severe hemorrhage and sepsis shock.

Anisodamine (ANI) is extracted from a Chinese herb *Anisodus tanguticus*. Because of its capability of improving the microcirculatory flow and splanchnic perfusion, ANI has been reported to work as an anti-shock medicine to treat burn shock, septic shock, and

ischemia shock (Sheng et al., 1997; Ruan et al., 2001; Hu and Sheng, 2002). However, the side effects of ANI such as dry mouth and accelerating heart rate (HR) have limited its wide clinical application. Therefore, penehyclidine hydrochloride (PHC), a new anticholinergic drug, was synthesized (Han et al., 2005). Compared to other anticholinergics, PHC is noticeable benefits for its selectively blocked M1, M3, N receptors, and few M2 receptor-associated cardiovascular side effects (Zhan et al., 2007). Recent experimental and clinical studies showed that PHC do not only improve microcirculation, reduced permeability of capillaries, decreased release of lysosomal enzymes (Han et al.,2005), but also was widely used in preoperative medication (Yan et al., 2006), and the treatment of organophosphate poisoning (Liang, 2007) .

AKI is a hazardous clinical issue correlated with high morbidity and mortality, and pharmacological therapy has been proven to be beneficial in some preclinical studies; however, the prevention and treatment of AKI is still highly unsuccessful (Ferenbach et al., 2010; Bajwa et al., 2009). There was no research concerning whether anticholinergic drug was effective to antagonise two-hits induced AKI. Regarding the AKI, characteristics of pathophysiology and histopathology are free radicals accumulation, inflammatory mediator activation, tubular necrosis, and glomerular damage, etc (Shah et al., 2010). The purposes of the present study were to determine whether anticholinergic drug: 1) decreased oxygen free radicals accumulation, 2) reduced inflammatory mediators and cytokine activation, 3) lessened kidney histopathological damages, and 4) ANI or PHC was more effective.

MATERIALS AND METHODS

Experimental animals

Forty-eight (48) Wistar rats (250 to 280 g) were randomly divided into four groups: sham group, two-hit (TH) group, TH treated with 1 mg/kg PHC (TH + PHC), and TH treated with 5 mg/kg ANI (TH + ANI). The doses of PHC and ANI were determined by the results of our preliminary experiment. All the rats were purchased from the Laboratory Animal Center of Southern Medical University. All experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of Southern Medical University.

Duplication of two-hits model

The two-hits model was duplicated by modified Zhou's method (Zhou et al., 2003). The rats were anesthetized with 20% urethane (5 ml/kg) by intramuscular injection. Under sterile conditions, the femoral artery was cannulated with 24-gauge tubing for blood exanguinating, mean arterial pressure (MAP) monitoring and blood sample collecting. The femoral vein was cannulated with 24-gauge tubing for liquid resuscitation and medicine administration. The first hit was initiated by blood withdrawal and reduction of the MAP to 35 ± 5 mmHg within 15 min. This blood pressure was maintained by further blood withdrawal if the MAP was >45 mmHg, and by infusion of 0.5 ml Ringer's lactate (RL) if the MAP was <30 mmHg. Shed blood was collected into 0.1 ml citrate/ml blood to prevent clotting. After a hypotensive period of 60 min, animals were resuscitated by transfusion of the shed blood and RL in a volume equal to that of shed blood over a period of 90 min. The second hit: in the end of resuscitation, LPS (2 mg/kg, *Escherichia coli* 0111:B4; Sigma, USA) was injected via femoral vein. Sham animals underwent the same surgical procedures, but neither the first hit nor second hit was performed. 1 h later, sham group and group TH received normal saline (NS) 5 ml/kg, while group TH + PHC and TH + ANI received 1 mg/kg PHC (LiSiTe Corporation, Chengdu, China) and 5 mg/kg ANI (KaiFeng Corporation, Henan, China), respectively (Figure 1). The blood samples were collected in 6 h. The plasma was drawn off. All the rats were sacrificed, and their kidney was removed. The plasma samples and the renal tissues after perfusion were frozen and stored at -80°C until assayed.

Measurement of superoxide dismutase (SOD) activity

Following the kit instructions (Nanjing Jiancheng Bioengineering Institute, China), stored renal tissues were homogenized in 100 mmol/L Tris-HCl buffer and centrifuged at 4,000 rpm/min for 15 min, and then the supernatant in each tube underwent colorimetric assay at 550 nm. The SOD activity was expressed as units per microgram of total protein (U/mg). Total protein content in samples was determined by a Coomassie blue protein assay kit.

Measurement of malondialdehyde (MDA) content

MDA was analyzed according to the kit instructions (Nanjing Jiancheng Bioengineering Institute, China). Stored renal tissue homogenate was centrifuged at 4,000 rpm/min for 15 min. Supernatant (0.15 ml) was transferred to a testing tube. A standard solution (0.15 ml) was added to each standard testing tube and 0.15 ml of distilled water was added to another blank testing tube, and then incubated at 100°C for 1 h. Finally, supernatant in each tube underwent colorimetric assay at 532 nm (Spectro- photometer Agilent 8453, USA). MDA content was expressed as nmol/mg protein.

Measurement of myeloperoxidase (MPO) activity

Renal MPO activity was analyzed by using MPO Detection Kit (Nanjing Jiancheng Bioengineering Institute, China). Stored tissue was homogenized in 1 ml of 50 mmol/L potassium phosphate buffered saline (PBS) containing 0.5% hexadecyl-trimethylammonium bromide and two freeze–thaw cycles. After the supernatant was added, the change in absorbance at 460 nm was observed for 10 min. MPO activity was expressed as U/g protein.

Blood biochemistry

Blood urea nitrogen (BUN) and plasma creatinine (Cr) concentrations were measured by using standard techniques with a serum analyzer (Olympus Automated Chemistry Analyzer AU400, Japan).

Measurement of tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) release

Liquidchip technology, a novel platform for clinical detection, because of its hypersensitivity and high efficiency, is being increasing popular in medical field. The essential of this technology is that polystyrene particles were (5.6 μm) stained and coded by

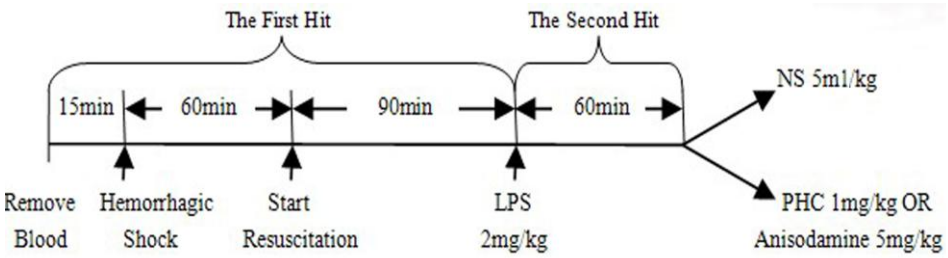

Figure 1. A schematic diagram showing the experimental protocol in the present studies. At the end of the resuscitation period, 2 mg/kg LPS was injected via the femoral vein. Sham animals underwent the same surgical procedures, but neither the first hit nor second hit was performed. 1 h later, sham group and group TH received NS 5 ml/kg, while groups TH + PHC and TH + ANI received 1 mg/kg PHC and 5 mg/kg ANI, respectively.

two fluorescent dye. These particles are irradiated by two separate lasers in the Luminex 100 analyzer (Qiagen, Germany). The machine distinguished the coded particles and detected the fluorescence intensity from the reporter molecules (Ren et al., 2011).

TNF-α, IL-1, and IL-6 levels of plasma were determined in a 96-well microtiter plate by using a Procarta Cytokine Assay Kit (Affymetrix, Inc, USA) according to the manufacturer's guidelines. Put the plate in Luminex 100 analyzer, the data acquisition was done with the integrated system software of the Liquidchip workstation, and data analysis with Microsoft Excel.

Histological examination of kidney

The renal specimens were fixed in 10% paraformaldehyde for 24 h and then embedded in paraffin, a series of microsections (5 mm) were stained with hematoxylin and eosin staining for light microscopy observation. The experimental conditions were examined with an inverted biological microscop (Eclipse Ti-S, Nikon, Japan). The severity of tissue damage was graded on a scale from 1 to 5. The different grades were defined as follows (Kong et al., 2010): grade 0 = no damage, grade 1 = minimal damage <10%, grade 2 = mild damage < 10 to 25%, grade 3 = moderate damage <25 to 50%, grade 4 = severe damage < 50 to 75%, and grade 5 = maximal damage >75%. The estimations were performed by_two blinded observers on coded sections (3 to 4 sections per kidney and 10 to 12 fields per section). The data are expressed as mean ±SD.

Immunohistochemical detection of intercellular adhesion molecule-1 (ICAM-1)

Paraffin sections were prepared by fixing the samples for 24 to 48 h in 10% buffered formalin. Following paraffin removal, samples were washed under flowing water and then reacted in 0.3% hydrogen peroxide for 15 min at room temperature to neutralize endogenous peroxidase activity. After washed in PBS for 15 min at room temperature, sections were incubated overnight at 4°C with mouse monoclonal antibody against ICAM-1 (1:50 dilution, Santa Cruz Biotechnologies, USA) followed by incubation with a biotinylated rabbit anti-mouse Immunoglobulin G (IgG). Diaminobenzidine tetrahydrochloride (Sigma, USA) was used as chromogen. Microscopy and image capture was carried out using a microscope with cooled charge coupled device (CCD) camera (Qicam, Olympus, Japan). The captured pictures were assessed by using the Image Pro Plus image 5.1 (Media Cybernetics, USA) analysis software system. Immunohistochemical staining mean density = integrated option density /positive area. The data are expressed as mean ±SD.

Statistical analysis

All assays were performed in duplicate, and the mean values were used for statistical analysis. The data are expressed as means ±SD and analyzed with SPSS statistical software package (SPSS 13.0; SPSS, Inc, Chicago, IL). Differences were determined by one-way analysis of variance followed by Fisher's least significant difference test. Kruskal-Wallis nonparametric test was performed for the histopathological analysis. The $P < 0.05$ values were considered significant.

RESULTS

The BUN and Cr levels of plasma

As shown in Figures 2A and 2B, The BUN and Cr levels of plasma exhibited significant raise on all the rats that underwent two-hits when compared with sham group (BUN:5.2 ± 1.8 pg/ml; Cr 53 ± 17.1 pg/ml; $P < 0.01$). BUN levels in groups TH + PHC (14.9 ± 1.85 pg/ml) and TH + ANI (17.7 ± 2.9 pg/ml) were found markedly lower than group TH (20.6 ± 3.6 pg/ml, $P < 0.05$), and group TH + ANI was not significantly higher than group TH + PHC ($P > 0.05$). Cr levels in group TH + PHC (83.25 ± 14.9 pg/ml, $P < 0.05$) and group TH + ANI (102.1 ± 18.5, $P>0.05$) were lower than group TH (122 ± 19.9 pg/ml). Cr levels in group TH + ANI were significantly higher than group TH + PHC ($P < 0.05$).

The SOD activities, MDA contents and MPO activities of renal tissue

SOD activities in renal tissues were investigated, and these data are shown in Figure 3A. SOD activities in all the rats experienced two-hits were significantly lower than sham group (220.7 ± 45.5 U/mg, $P < 0.01$ or $P < 0.05$). Compared with group TH (99.8 ± 21.2 U/mg), treatment with 1 mg/kg PHC (159.1 ± 23.7 U/mg, $P < 0.01$) and 5 mg/kg ANI (127.7 ± 18.1 U/mg, $P < 0.05$) significantly prevented reduction in SOD activities. The data of MDA contents and MPO activities are shown in Figures 3B and 3C. Significantly increased MDA contents

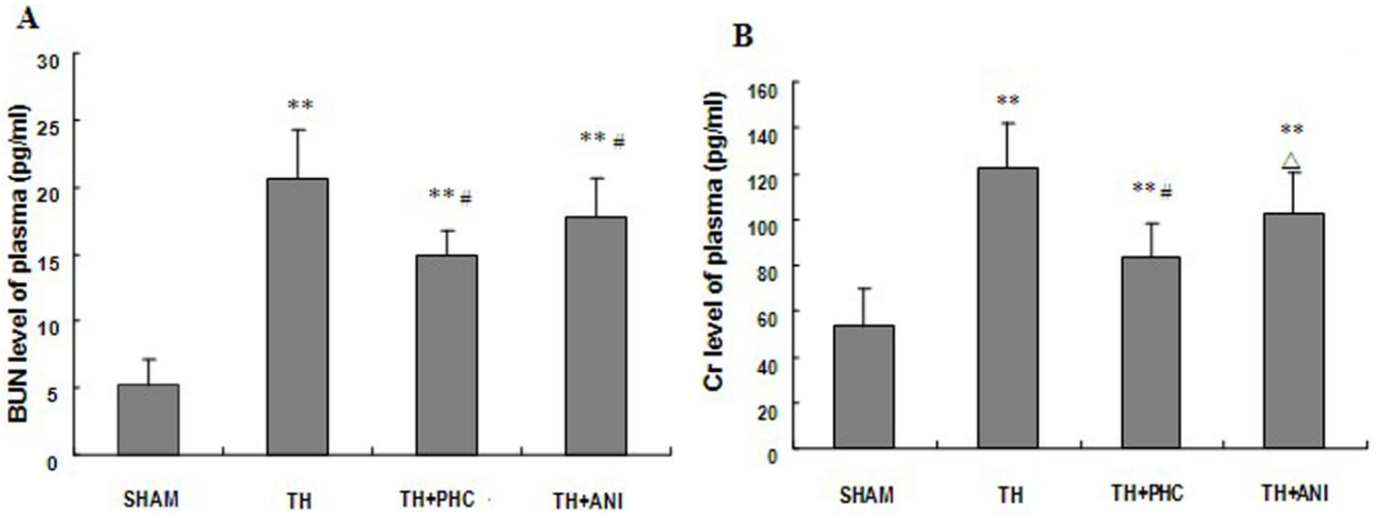

Figure 2. The levels of BUN and Cr of plasma in four groups. Data presented means ±SD. **P<0.01 versus sham group; #P < 0.05, ##P <0.01 versus group TH; △P < 0.05 versus group TH + PHC.

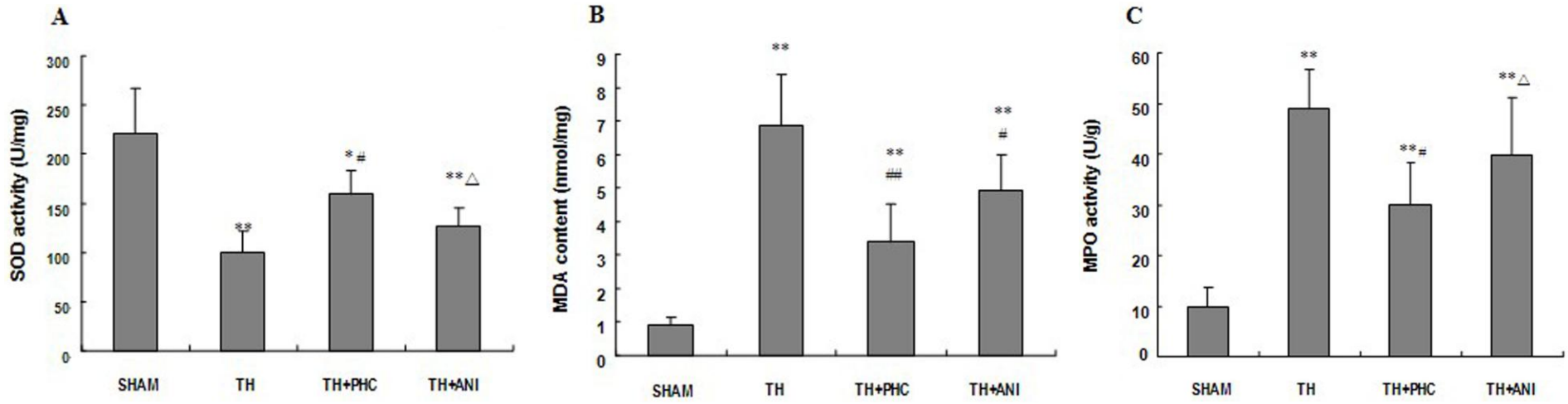

Figure 3. The renal tissue SOD activities, MDA contents and MPO activities in four groups. (A) SOD; (B) MDA; (C) MPO. Data presented means ±SD. *P < 0.05, **P <0.01 versus sham group; #P < 0.05, ##P <0.01 versus group TH; △P < 0.05 versus group TH+ PHC.

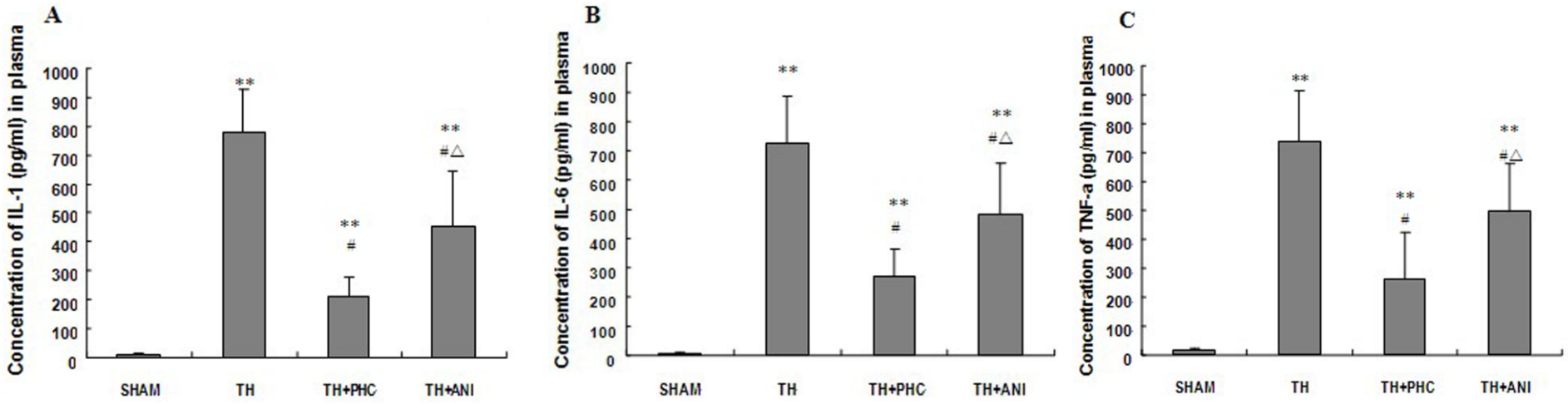

Figure 4. Concentration of TNF-α, IL-1 and IL-6 of plasma in four groups. (A) IL-1; (B); IL-6; (C) TNF-α. Data presented means ±SD. **P < 0.01 versus sham group; #P < 0.05, ##P <0.01 versus group TH; △P < 0.05 versus group TH + PHC.

and MPO activities in all the rats that experienced two-hit were observed when compared with sham group (MDA: 0.92 ± 0.24 nmol/mg, MPO: 9.9 ± 3.6 U/g, P < 0.01). Compared with TH group (6.8 ± 1.5 nmol/mg), treatment with 1mg/kg PHC (3.4 ± 1.1 nmol/mg, P < 0.01) and 5 mg/kg ANI (4.9 ± 1.1 nmol/mg, P < 0.05) decreased MDA contents. MPO activities in group TH + PHC (29.9 ± 8.4 U/g, P < 0.05) and group TH + ANI (39.9 ± 11.2 U/g, P > 0.05) were lower than group TH (48.7 ± 7.9 U/g). MDA content in group TH + PHC was lower than group TH + ANI, but no significant difference was found in these two groups (P > 0.05). On the other hand, MPO activity in group TH + PHC was lower than group TH + ANI (P < 0.05).

The concentrations of,IL-1, IL-6, TNF-α in plasma

Figure 4 shows the data of IL-1 (Figure 4A), IL-6 (Figure 4B) and TNF-α (Figure 4C) concentration in plasma. Compared with sham group (IL-1: 9.8 ± 3.1 pg/ml; IL-6: 16.8 ± 7.4 pg/ml; TNF-α: 6.4 ± 3.7 pg/ml), the concentrations of IL-1, IL-6, and TNF-α (P < 0.01) in groups suffered two-hits were significantly elevated. Group TH + PHC and group TH + ANI notably lessened the concentrations of IL-1, IL-6, and TNF-α in plasma when compared with group TH (IL-1:781.3 ± 145.1 pg/ml; IL-6:723.9 ± 163.9 pg/ml; THF-α: 736.2 ± 177.1 pg/ml; P < 0.05). The concentrations of IL-1 (453.1 ± 193.7 pg/ml), IL-6 (481.2 ± 176.9 pg/ml) and TNF-α (494.2 ± 166.8pg/ml) in plasma in group TH + ANI were notably higher than group TH + PHC (IL-1: 211.3 ± 67.8 pg/ml; IL-6: 273.7 ± 90.1 pg/ml; THF-α: 262.4 ± 162.1 pg/ml, P < 0.05).

Histological examination

The structure of the renal glomeruli and tubuli, proximal and distal convoluted tubes were seen clearly in the sham group (Figure 5A). Severe tubular dilatation and obstruction, glomerulus sclerosis and necrosis, and the development of protein casts were presented in the kidney of the rats in group TH (Figure 5B). Patchy glomeruli and interstitial hemorrhage, tubular epithelium swelling, were found in group TH + PHC (Figure 5C) and group TH + ANI (Figure 5D), the damage severity of renal tissues in these two groups (TH + PHC: 9.8 ± 1.2; TH + ANI:18.1 ± 1.2) were significantly lower than group TH (27.7 ± 1.2, P < 0.05). The mean grade scored of the rats treated with PHC was lower than the rats treated with ANI (P < 0.05).

Immunohistochemical detection of ICAM-1

Figure 6 indicates the representative immunohisto-chemical detections of ICAM-1

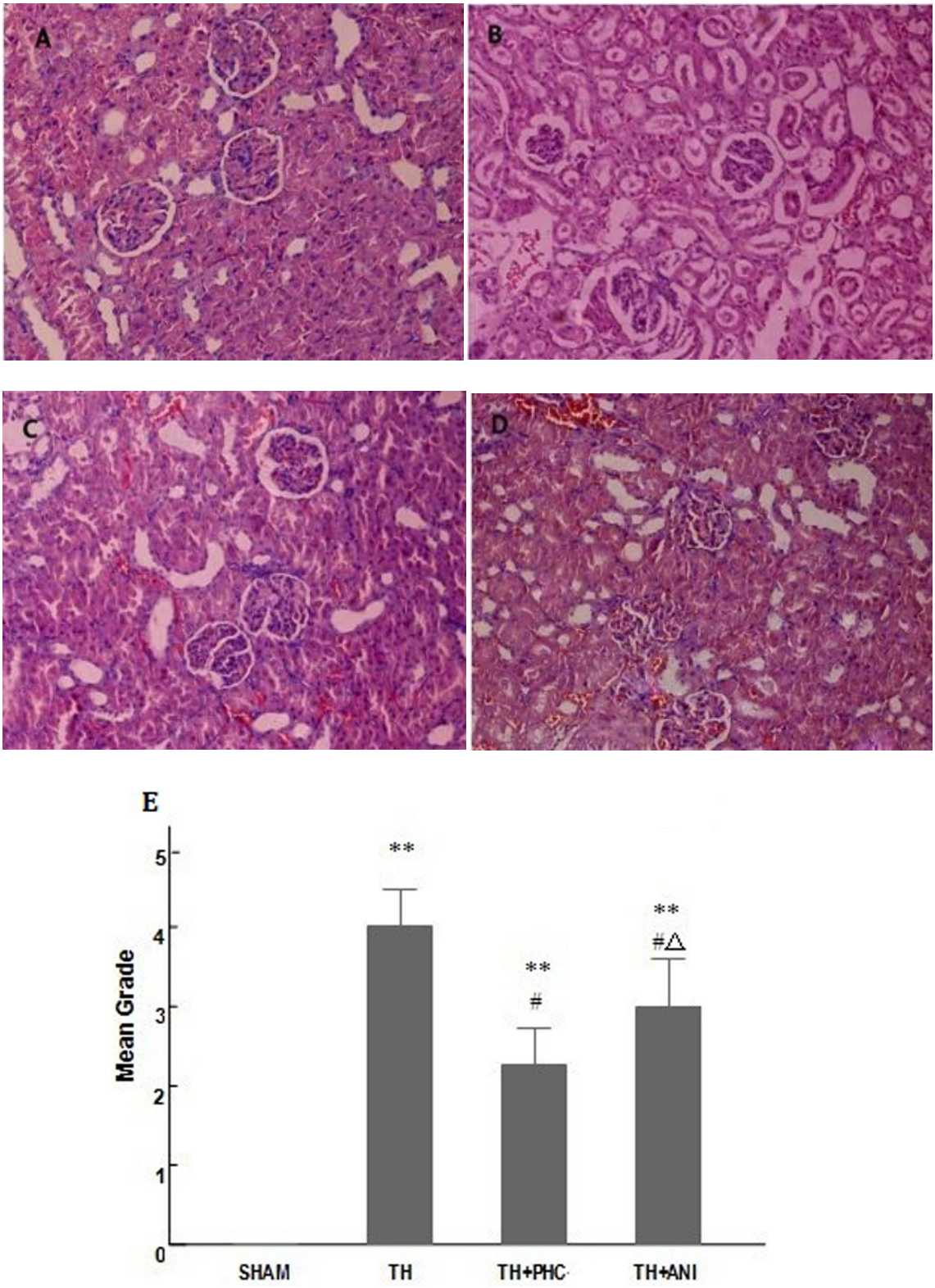

Figure 5. Representative photomicrographs of kidneys. (A) Regular morphology with glomeruli and tubuli were seen in the sham group; (B) glomeruli and tubuli were seriously damaged in the group TH; (C) damages relieved in animals treated with PHC; (D) damages relieved in animals treated with ANI; Original magnification: ×200; (E) Mean grading scale of histological appearance of sham group, group TH, and groups treated with PHC or ANI after TH. **P < 0.01 versus sham group; $^{\#}$P < 0.05 versus group TH; $^{\triangle}$P < 0.05 versus group TH + PHC. Data are presented as means ±SD.

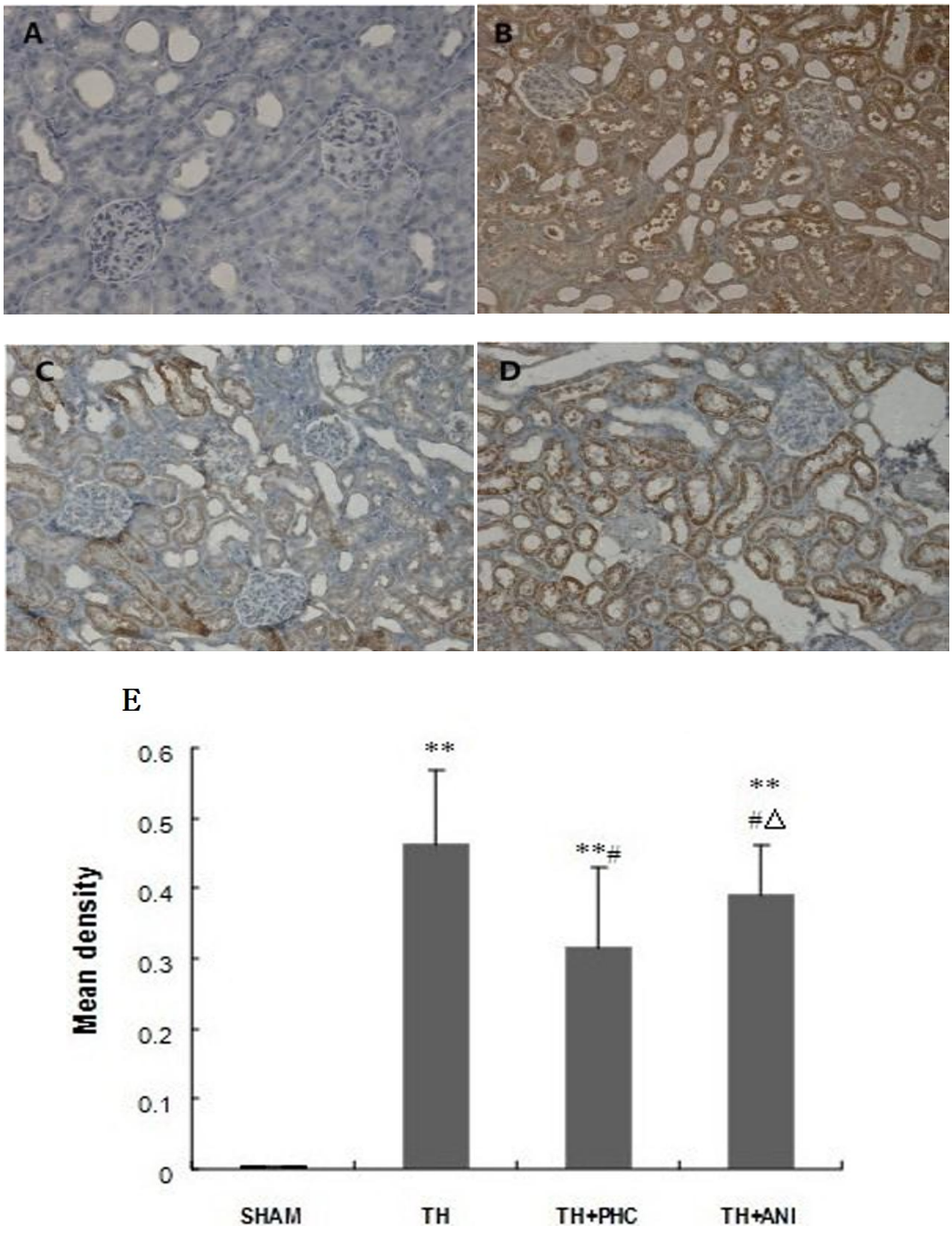

Figure 6. Representative photomicrographs of immunohistochemical detection of ICAM-1 expression in (A), sham group; (B), TH group; (C), TH + PHC group; (D), TH+ANI group. Immunohistochemical staining mean density = integrated option density /positive area. (E), Mean density of ICAM-1 expression of sham group, TH group, groups treated with PHC or ANI after TH. **P < 0.01 versus sham group; #P < 0.05 versus group TH; △P < 0.05 versus group TH + PHC. Data are presented as means ±SD.

expression in renal tissues. Sham rats did not exhibit detectable levels of ICAM-1 expression (Figure 6A). Rats subjected to TH showed a notably increased ICAM-1 expression (Figure 6B, 0.46 ± 0.11, P < 0.01). Compared with group TH, ICAM-1 expression in groups TH + PHC (0.31 ± 0.12, P < 0.05) and TH + ANI (0.39 ± 0.07, P < 0.05) were notably decreased. ICAM-1 expression in group TH + PHC (Figure 6C) were lower than group TH

+ ANI (Figure 6D, $P < 0.05$).

DISCUSSION

Inflammation after renal IRI is not only a major contributor of renal cell death, but also a potential mechanism to initiate and maintain renal cell necrosis and apoptosis (Saadia and Schein, 1999). Aregger et al. (2009) did not consider blood loss which was the main cause of AKI in multivariate analysis; however, SIRS after transfusion was a high risk factor and the possible mechanism of AKI. Since AKI has a high morbidity and mortality, the study of prevention and therapy required an animal model truly replicated the complex pathogenesis of human AKI. In the present study, two-hit model was adopted to present a similar pathogenesis, pathology, and complexity of the patients suffering AKI that was secondary to trauma, hypotension shock and severe infection.

As we know, inflammation process involves multiple inflammatory mediators, neutrophils recruitment, and macrophages infiltration. Neutrophils rapidly respond to injury and release MPO and proteolytic enzymes, which generate reactive oxygen species. Macrophages produce pro-inflammatory cytokines that can stimulate the activity of other leukocytes. Analysis of kidney infiltrating macrophages demonstrated that these leukocytes were the major producer of the cytokines IL-1, IL-6, and TNF-α (Bajwa et al., 2009). TNF-α has been proven to play a "master-regulator" role in orchestrating the cytokine cascade in many inflammatory diseases (Parameswaran and Patial, 2010). IL-1 and IL-6 have been reported as good indicators of activation of cytokine cascade in various conditions (Oda et al., 2005). These pro-inflammatory cytokines were essential during the early phase of IRI, inflammation, and endotoxemia (Ishii et al., 2010; Lloyd et al., 2003). Therefore, it is important to inhibit the release of IL-1, IL-6, and TNF-α for lessening inflammatory responses. The present study found the plasma concentrations of IL-1, IL-6, and TNF-α in group TH was significantly higher than that in the groups treated with anticholinergics, the data demonstrated that PHC and ANI acted positively to inflammatory response. The protection against two-hits induced AKI by PHC and ANI can probably be ascribed to the reduced generation of IL-1, IL-6, and TNF-α generated by macrophages.

MPO is an enzyme mainly located in the primary granules of neutrophils; the tissue MPO levels may indicate neutrophils infiltration (Tuğtepe et al., 2007). MDA, a lipid peroxidation end product, is widely worked as a marker of oxidative stress (Gaweł et al., 2004). According to our findings, PHC and ANI treatments significantly suppressed the activity of MPO and the MDA contents in renal tissue, which illustrated that anticholinergics treatment could inhibit the infiltration of neutrophils into renal parenchyma or medulla nephrica and attenuate oxidative stress. SOD is an enzyme that exists in cells removing oxyradicals, whose activity variation may represent the degree of tissue injury (Macarthur et al., 2000). The present study also revealed that SOD activities in group TH were remarkably lower than the groups treated with PHC and ANI. These findings implied that the feature of anticholinergics are to reduce MDA contents and enhance SOD abilities, which prevented renal tissue from cellular membrane destroy and chondriosome dysfunction attacked by oxygen free radicals.

ICAM-1 is found abundantly in endothelial, epithelial, and mesangial cells and fibroblasts. It is up-regulated *in vitro* and *in vivo* by cytokines such as TNF-α and IL-1 (Burne et al., 2001). Park et al. (1008) reported that IL-1 and TNF-α could regulate the expression of leukocyte-binding adhesion molecules in endothelial cells derived from human glomerulus. Our study showed that ICAM-1 expression decreased significantly in the groups treated with anticholinergics while it was compared with the group TH. These data suggested that ICAM-1 played a significant role during the neutrophil dependent injury phase after renal ischemia and reperfusion. As a result, suppressing adhesion molecule may have potential to against AKI induced by two hits.

In our study, we also found that the plasma BUN and Cr levels in group TH were significantly higher than that in groups treated with anticholinergics. A histopathological observation indicated that the cellular structures of the kidney in the sham group were normal, while congestion, degeneration and necrosis were found in the group TH, and mild lesions were found in the groups treated with PHC and ANI. The pathologic changes of group TH were increased infiltration of neutrophils, glomerular sclerosis, tubular obstruction and necrosis. The glomerulus and tubular damage degree of PHC and ANI treatment groups were alleviated when compared with group TH. It suggested that anticholinergics had a beneficial effect on the kidney in two-hit rats.

ANI, a classical anticholinergic, has been prescribed for the treatment of certain diseases such as COPD, Alzheimer disease, and urinary incontinence for its capability to relieve small blood vessel spasm and improve microcirculation (Han et al., 2005). However, the side effects such as accelerating HR and short effective drug duration have restricted its clinical application. M2 receptors are distributed in the atrial myocardium. Liang et al. (2008) reported a potential value of M2 receptor antagonists in the treatment of certain types of arrhythmia and atrial fibrillation. The new traits of PHC are selective blocking M1 and M3 receptors, faster reaction time and long effective drug duration, making PHC having less cardiovascular side effects such as sychnosphygmia and arrhythmia than ANI. Therefore, PHC could reduce myocardial consumption of oxygen and heart burden. The improvement of cardiac preload and cardiac function is profited to antagonize hemorrhagic shock and sepsis

shock. The features of PHC can explain the information we found in the present study. Cr and BUN levels, oxidative stress products, and inflammatory cytokines in TH + PHC group were lower than group TH + ANI. PHC treatment in two-hits induced rats AKI can result in a better therapy than ANI by attenuating cytokines activation, lessening renal histopathological damage, and decreasing oxygen free radicals accumulation.

Conclusion

The investigation found that both PHC and ANI had beneficial effects on two-hits induced rats AKI, whose underlying mechanism was probably to inhibit the production of inflammatory cytokines IL-1, IL-6, and TNF-α, stabilize the cell membrane and resist oxygen free radicals, attenuate neutrophils recruitment and macrophages infiltration, and suppress the expression of ICAM-1 in renal tissue. It is the first time these findings are reported with regard to AKI. This study may provide a novel strategy for the clinical physician against two-hit induced AKI.

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