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ORIGINAL RESEARCH

Short–Term Preconditioning with Insulin and Glucose Efficiently Protected the Kidney Against Ischemia-Reperfusion Injury via the P-AKT-Bax-Caspase-3 Signaling Pathway in Mice

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Objective: Insulin attaches insulin receptor to activate the PI3-kinase/Akt signaling to maintain glucose homeostasis and inhibit apoptosis. This study determined whether preconditioning with insulin and glucose protects the kidney against ischemia-reperfusion injury (IRI).

Methods: Kidney IRI was performed in C57BL/6 mice by clamping the renal vessels for 30 min, followed by reperfusion for 24 h. A total subcutaneous 0.1 unit of insulin along with 10% glucose in drinking water was treated on the mice for 24 h before kidney IRI. The kidney function and injuries were investigated through the determination of BUN and Cr in blood plasma, as well as the apoptosis and the expression of P-AKT, BAX, and caspase-3 in the kidneys. The role of P-AKT in insulin-treated IRI kidneys was tested using an AKT inhibitor. The effects of the preconditional duration of insulin and glucose on IRI kidneys were investigated by expanding the treatment duration to 1, 3, and 6 days.

Results: Preconditioning with insulin and glucose protected the kidney against IRI as manifested by a decrease in creatinine and BUN and a reduction of kidney tubular injury. The protection effect was mediated by P-AKT-BAX-caspase-3 signaling pathway resulting in suppression of apoptotic cell death. An AKT inhibitor partially reversed the protective effects of preconditional insulin. The preconditional duration for 1, 3, and 6 days had no differences in improving kidney functions and pathology.

Conclusion: A short-term preconditioning with insulin and glucose protected the kidney from IRI through the activation of p-AKT and subsequent reduction of BAX-caspase-3-induced apoptosis. The short-term precondition provides a practicable strategy for protecting the kidney against predictable IRI, such as kidney transplant and major surgical operations with high risk of hypotension.

Keywords: kidney, insulin, ischemia-reperfusion injury, AKT, Bax, caspase-3

Introduction

Acute kidney injury caused by ischemia-reperfusion is a common condition in multiple clinical settings such as septic shock, trauma, kidney transplantation, and cardiovascular surgery.[1–3] Acute kidney injury could lead to high mortality and a lengthy ICU stay.[3,4] Aggressive prevention of acute kidney injury remarkably reduces mortality and medical expenses for critically ill patients.[5] Insulin has been shown to prevent human renal tubular epithelial cells from camptothecin-induced apoptosis through the activation of the PI3-kinase/Akt pathway.[6] Insulin attaches insulin receptor to activate the PI3-kinase/Akt/AS160(or GSK3) signaling to maintain glucose homeostasis. Subsequently phosphorylated AS160 translocate GLUT4 to cellular membrane to uptake glucose and phosphorylated GSK3 increased glycogen synthesis.[7] The insulin/insulin receptor/PI3-kinase/Akt signaling

Received: 5 April 2024
Accepted: 13 June 2024
Published: 20 June 2024

was also found inhibition of hypoxic-ischemic-induced apoptosis through inhibiting autophagy and endoplasmic reticulum stress.[8]

Therefore, insulin is usually used to preserve the vitality of renal tubular epithelial cells in culture.[9] In rats with diabetes mellitus (DM), insulin treatment before ischemia-reperfusion injury (IRI) preserved both renal function and histomorphology, whereas insulin treatment after IRI had no such protective effects.[10] Fei Tong et al reported that preconditioning with insulin improved the recovery of renal dysfunction in DM rats after kidney IRI.[11] In a normal glucose animal model, 96 h of glucose-insulin infusion before and after IRI was found to protect the kidney against IRI. However, the glucose-insulin infusion for such a long time in rats is difficult to translate into clinical practice. This study did not confirm whether the glucose-insulin infusion before IRI protects the kidney against IRI. Moreover, this experiment did not explore how insulin and glucose prevent ischemic-reperfusion-induced kidney damage.[12] In the clinical setting, intensive insulin therapy in severely ill patients has been found to reduce the incidence of acute kidney injury by 38% and reduce the need for dialysis by 35%. Meanwhile, intensive insulin therapy increased the risk of hypoglycemia fourfold compared with conventional therapy.[13,14] There is also a contradictory finding about the renal effects of intensive insulin therapy or intensive glucose control. A meta-analysis discovered that intensive glucose control with a target glucose goal of <6.1 mmol/L (110 mg/dL) had no effect to decrease mortality rate or dialysis requirement in severely ill adult patients but increased the incidence of hypoglycemia.[15] In a two-center, randomized controlled trial, tight glycemic control was found to reduce nosocomial infection in the postoperative period following pediatric cardiac surgery.[16] The secondary analysis of this study revealed that tight glycemic control had no effect on the reduction of cardiac surgery-associated acute kidney injury rate.[17,18] Although the renal protection of insulin is not yet conclusive, it is still worthwhile to investigate the use of insulin to prevent IRI in the kidney, given that insulin treatment is clinically feasible. Insulin treatment after operation may cause serious hypoglycemia. Insulin treatment before IRI may be more convenient and has little effect on the recovery from IRI. The combination of insulin and glucose may decrease hypoglycemia incidents. Therefore, we hypothesize that preconditioning with insulin and glucose could protect kidney from IRI. The present study determined whether preconditioning with insulin and glucose could prevent renal IRI in mice. Moreover, the possible mechanism of insulin in kidney protection was investigated.

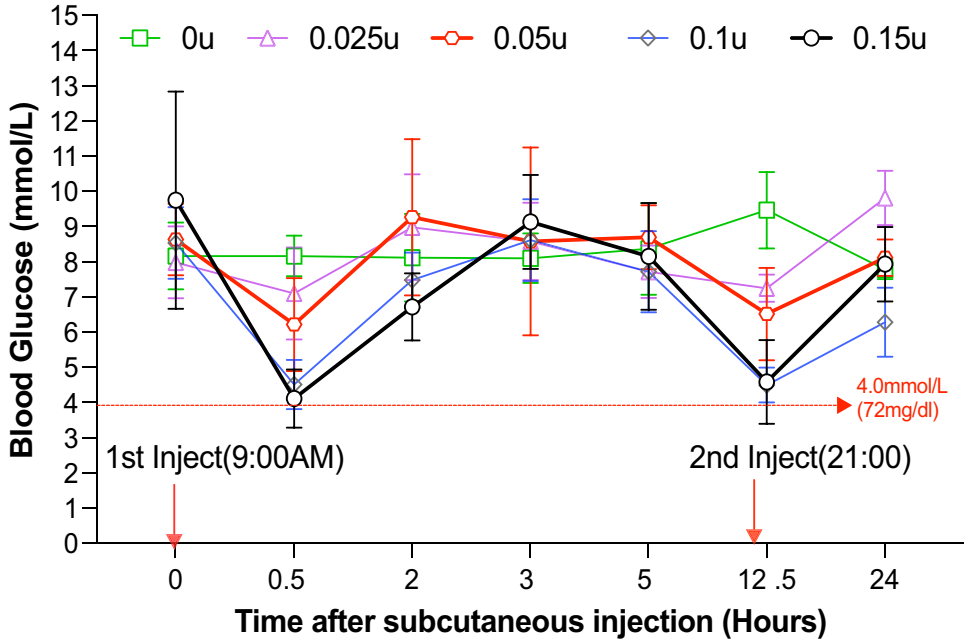

Figure 1 The blood glucose concentration changed over time after the subcutaneous injection of insulin in mice supplied with 10% glucose drinking water. Hypoglycemia was defined as a blood glucose concentration below 4 mmol/L (72 mg/dL) in this study. N=6 in each group.

Methods

Animals

The present study was approved by the ethics committee of Zhengzhou Central Hospital of Zhengzhou University. The Guidelines of Laboratory Animal Care were strictly followed in animal experiments. Wild-type (WT) C57BL/6 male mice, 24–26 g, were commercially obtained from the animal center of Zhengzhou University. The mice were acclimatized for 2 days before experiment in our lab. The 10% glucose drinking water was supplied to the mice during the whole study. Blood glucose concentration from the tail tip was monitored using a blood glucose meter (Sinocare Inc., Changsha, China). The first dose of insulin (Insulin glargine injection, Solostar, Beijing, China) was subcutaneously injected in the thigh region of the hindlimbs of the mice at 9:00 a.m. and the second dose of insulin was injected 12h later. The vehicle-treated mice were subcutaneously injected with the same volume of normal saline as the insulin injection. Mice were subjected to renal IRI at 9:00 a.m., as previously reported.[19] Briefly, anesthesia was performed by intraperitoneal injections of esketamine (50 mg/kg) and dexmedetomidine (0.25 mg/kg).[20] The kidneys were exposed and subjected to ischemia by clamping renal pedicles with non-traumatic micro-aneurism clamps. The clamps were removed after 30 min of ischemia. The mice were kept warm throughout the procedure on a heating pad. Sham-control mice were subjected to the same surgical procedure except for pedicle clamping.

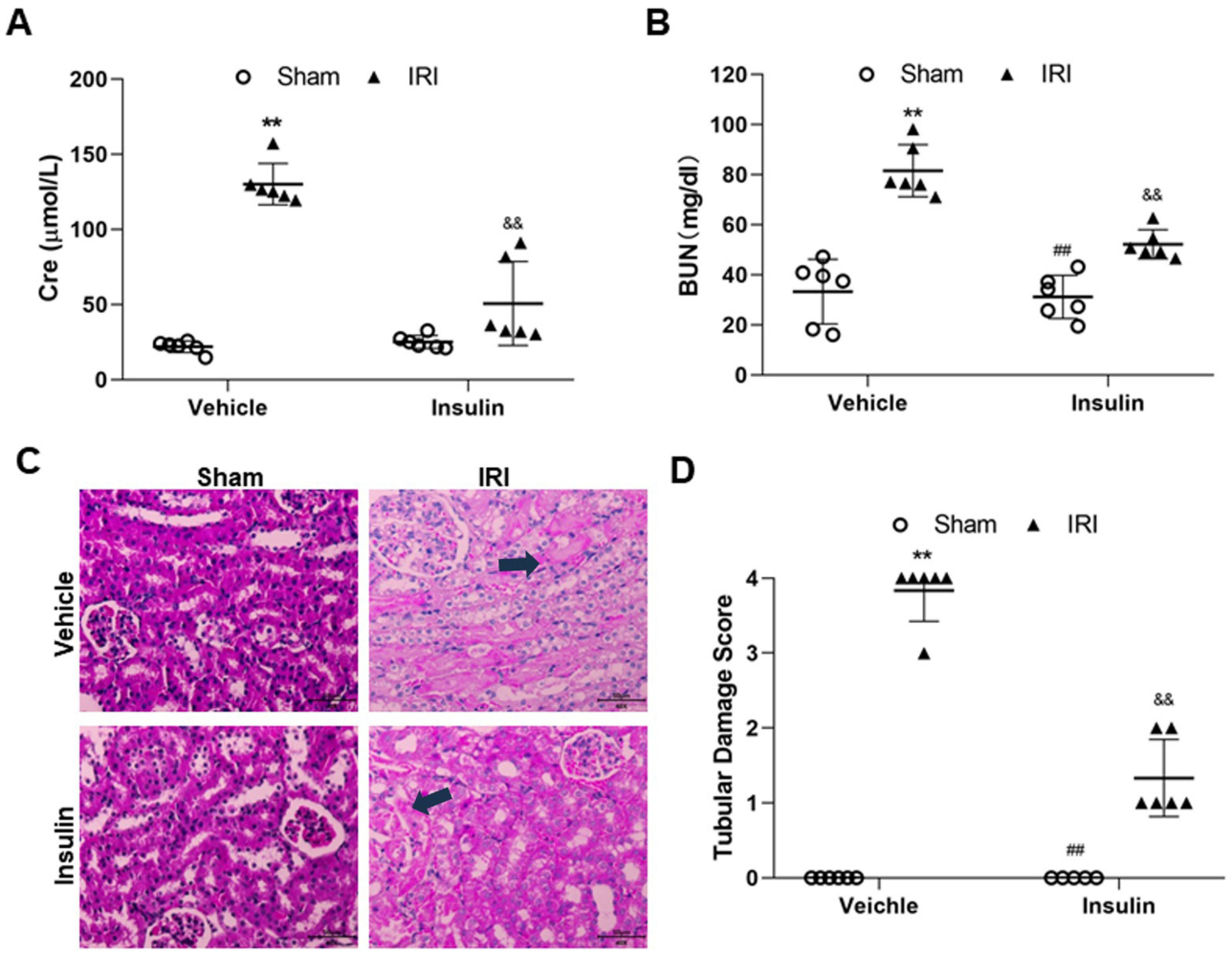

Figure 2 Preconditional 0.05-unit insulin injection and 10% glucose drinking water protected the kidney from IRI. (**A**) Effect of insulin and glucose on serum Cre concentration among the four groups (Sham surgery with vehicle injection, IRI surgery with vehicle injection, Sham surgery with insulin injection, and IRI surgery with insulin injection). **P < 0.01, compared with the vehicle-treated sham group. $^{\&\&}$P < 0.01, compared with the vehicle-treated IRI group. (**B**) Effect of insulin and glucose on serum BUN concentration among the four groups. **P < 0.01, compared with the vehicle-treated sham group. $^{\#\#}$P < 0.01, compared with the insulin-treated IRI group. $^{\&\&}$P < 0.01, compared with the vehicle-treated IRI group. (**C**) Representative H&E staining images of the kidneys from the four groups. The black arrows indicate the necrosis of tubular epithelial cells which form cast in tubular lumen without cellular shape. (**D**) Tubular damage score of the four groups. **P < 0.01, compared with the vehicle-treated sham group. $^{\#\#}$P < 0.01, compared with the insulin-treated IRI group. $^{\&\&}$P < 0.01, compared with the vehicle-treated IRI group. N=6 in each group.

The mice were sacrificed 24 h after reperfusion, and their kidneys were then harvested for Western Blot analysis and immunohistochemistry (IHC). For the pharmacological study, AKT inhibitor at 40 mg/kg (3-[1-[[4-(7-phenyl-3H-imidazo [4, 5-g] quinoxalin-6-yl) phenyl] methyl] piperidin-4-yl]-1H-benzimidazol-2-one, Beyotime, China) or vehicle was administrated intraperitoneally 4 h before IRI or sham control mice.

In this study, each experiment was repeated biologically for 6 times and each repeat was performed in a separate, independent way. Firstly, the treatment insulin dose was determined by detecting the whole blood glucose level. Secondly, the changes of insulin-AKT-Bax-Caspase 3-apoptosis signaling were analyzed in the ischemia-reperfusion kidney. Thirdly, the insulin-AKT-Bax-Caspase 3-apoptosis signaling was confirmed by AKT inhibitor. Lastly, the effects of the extension of the preconditioning period were also tested in the ischemia-reperfusion kidney.

Kidney Function

Serum creatinine was measured using a commercially available creatinine assay kit (Cat No.C011-2-1, Nanjing Jiancheng Bioengineering Institute). Blood urea nitrogen (BUN) (Cat No. BC1535, Solarbio, China) was measured as previously described.[21]

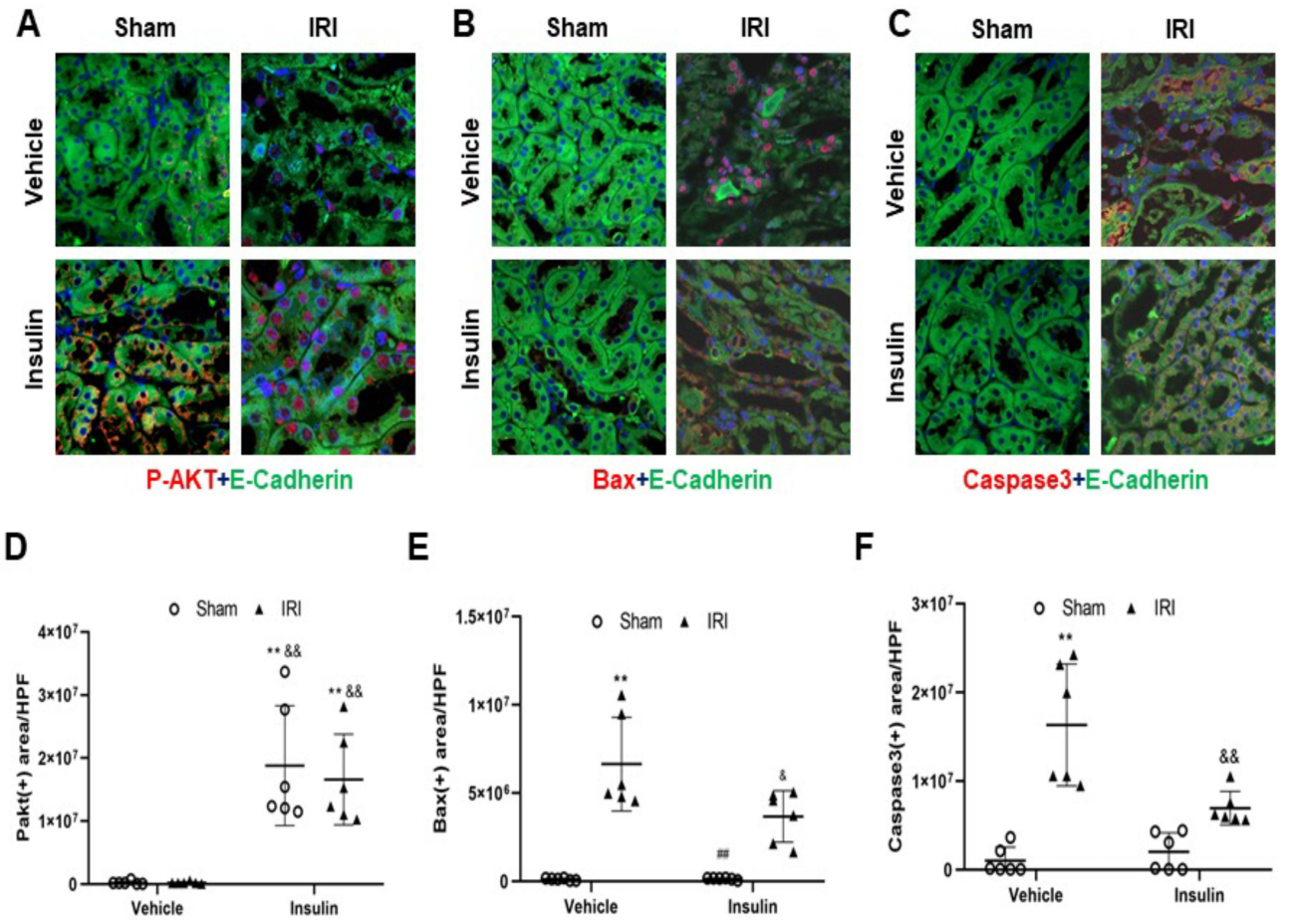

Figure 3 Immunofluorescence staining for P-AKT, Bax, and caspase-3 in the kidneys. (**A**) P-AKT expression in the kidneys of the four groups (Sham surgery with vehicle injection, IRI surgery with vehicle injection, Sham surgery with insulin injection, and IRI surgery with insulin injection). P-AKR was stained red with Cy5.5. E-Cadherin was stained green with FITC in the cytoplasm of tubular epithelial cells. The nucleus was stained purple with DAPI. (**B**) BAX expression in the kidneys of the four groups. BAX was stained red with Cy5.5. E-Cadherin was stained green with FITC. The nucleus was stained purple with DAPI. (**C**) Caspase-3 expression in the kidneys of the four groups. Caspase-3 was stained red with Cy5.5. E-Cadherin was stained green with FITC. The nucleus was stained purple with DAPI. (**D**) Quantitative analysis of P-AKT expression in the kidneys of the four groups. $^{**}P < 0.01$, compared with the vehicle-treated sham group. $^{\&\&}P < 0.01$, compared with the vehicle-treated IRI group. (**E**) Quantitative analysis of BAX expression in the kidney of the four groups. $^{**}P < 0.01$, compared with the vehicle-treated sham group. $^{\#\#}P < 0.01$, compared with the insulin-treated IRI group. $^{\&}P < 0.05$, compared with the vehicle-treated IRI group. (**F**) The quantitative analysis of caspase-3 expression in the kidney of the four groups. $^{**}P < 0.01$, compared with the vehicle-treated sham group. $^{\&\&}P < 0.01$, compared with the vehicle-treated IRI group. N=6 in each group.

Histological Analysis of Kidney Damage

Kidney tissues were immerged in 10% buffered formalin for fixation for at least 24 hours. Subsequently, the fixed kidney was embedded in paraffin and cut into 4-μm-thick sections. Then the sections were stained with hematoxylin and eosin after deparaffinization and rehydration. Tissue damage was scored according to the percentage of damaged tubules: 0, no damage; 1, 1–25% damage; 2, 25–50% damage; 3, 50–75% damage; and 4, 75–100% damage, as previously reported.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded kidney sections.[15] Antigen retrieving solution (0.01M sodium citrate, Ph. 6.0, Solarbio, life sciences, China) was used to retrieve the antigen from fixation. Three percent H_2O_2 was used to block endogenous peroxidase activity before blocking. After incubation with 5% normal serum, the slides were incubated with primary antibodies in a humidified chamber overnight. After washing, kidney sections were incubated with secondary antibodies, followed by ABC solution as previously reported.[16] Diaminobenzidine solution was applied to visualize the secondary antibody conjugated with horseradish peroxidase (HRP). The red substrate was used to visualize the secondary antibody conjugated with alkaline phosphatase (AP). Hematoxylin was used for nuclear staining. The sections were dehydrated with ethanol, cleared with histoclear, and then mounted using a mounting medium. NIH

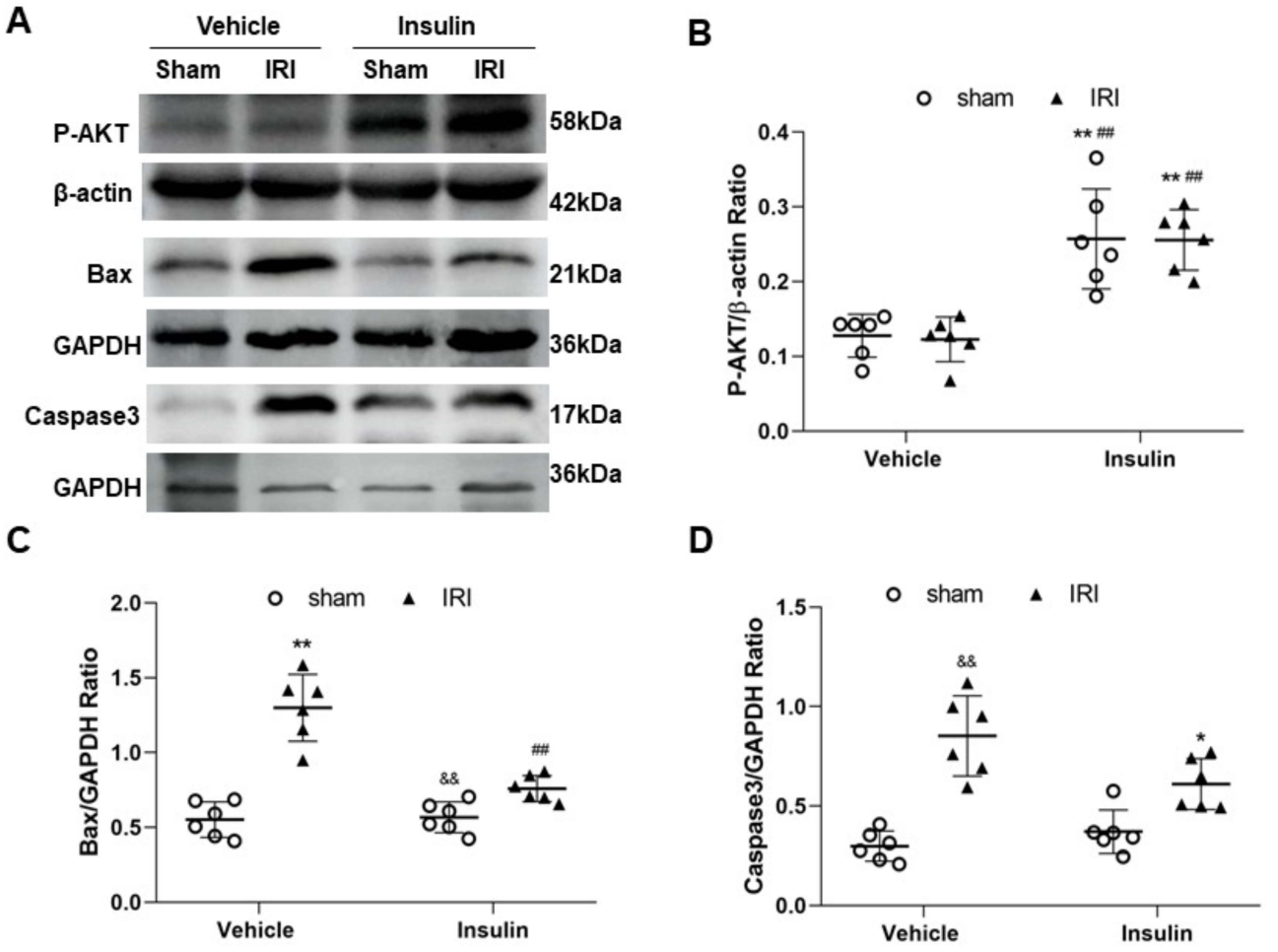

Figure 4 Western blot results of P-AKT, Bax, and caspase-3 in the kidneys. (**A**) Representative results of P-AKT, Bax, and caspase-3 expression in Western blot results of the four groups (Sham surgery with vehicle injection, IRI surgery with Vehicle injection, Sham surgery with Insulin injection, and IRI surgery with insulin injection). (**B**) Quantitative analysis of P-AKT expression in the Western blot. **P < 0.01, compared with the vehicle-treated sham group. ##P < 0.01, compared with the vehicle-treated IRI group. (**C**) Quantitative analysis of Bax expression in the Western blot. **P < 0.01, compared with the vehicle-treated sham group. &&P < 0.01, compared with the insulin-treated IRI group. ## P < 0.01, compared with the vehicle-treated IRI group. (**D**) Quantitative analysis of cleaved caspase-3 expression in the Western blot. &&P < 0.01, compared with the vehicle-treated sham group. *P < 0.05, compared with the vehicle-treated IRI group. N=6 in each group.

Image/J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the protein expression levels in the kidney.

Apoptosis Detection

ApopTag plus Peroxidase in situ Apoptosis Detection Kit (Cat no. MK1025, Booster Biological Technology, Ltd. China) was used to detect apoptosis. The apoptotic cells were determined using a terminal transferase dUTP nick-end labeling (TUNEL) assay. The numbers of TUNEL-positive cells per high-power field were quantified in a blinded manner.

Western Blot Analysis

The proteins were extracted using RIPA buffer containing a cocktail of protease inhibitors. The extracted protein concentration was determined using a Bio-Rad protein assay.[21,22] Approximately 40 μg of protein was loaded onto SDS-polyacrylamide gels in a Tris/SDS buffer system. The proteins in gels were then transferred onto nitrocellulose membranes. After blocking, the membranes were incubated with primary antibodies (anti-P-AKT(Proteintech, 80455-1-RR, USA. Diluted at 1:1000), anti-Bax(Proteintech, 50599-2-IG, USA. Diluted at 1:1000), anti-Caspase 3(Abbkine, ABP50855, China. Diluted at 1:1000)) overnight. After washing, the membranes were incubated with fluorescence-conjugated secondary antibodies. The proteins were detected using a Western blot imaging system (Amersham™ ImageQuant™ 800 biomolecular imager, MA, USA) which was commercially obtained in our lab. NIH Image/J software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the protein expression.

Statistical Analysis

All data are expressed as mean ± SD except tubular damage scores, which were presented as median with interquartile range. ANOVA was used to compare multiple groups followed by the Bonferroni procedure to compare means. Tubular damage scores were analyzed using the Wilcoxon rank sum test. $P < 0.05$ was considered statistically significant.

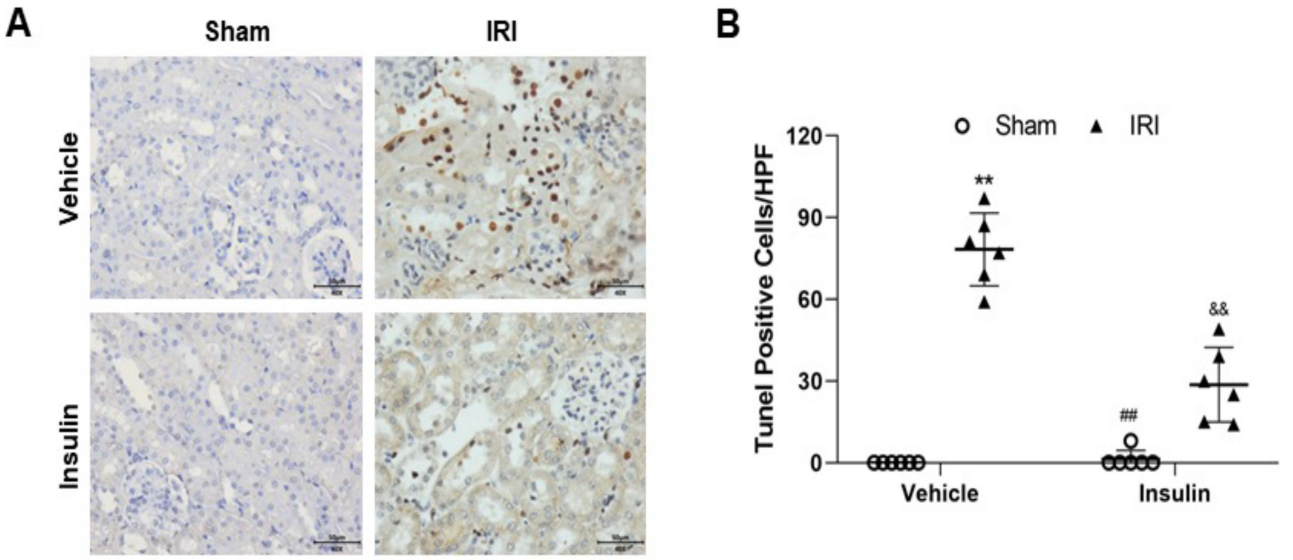

Figure 5 Apoptosis in the kidney after IRI using TUNEL assay. (**A**) Representative results of apoptosis in the kidneys with apoptotic cells (brown) counterstaining with hematoxylin (blue) of the four groups (Sham surgery with vehicle injection, IRI surgery with vehicle injection, Sham surgery with Insulin injection, and IRI surgery with insulin injection) (original magnification is 400×). (**B**) The apoptotic cells were quantitatively analyzed in the kidneys. **P < 0.01 compared with the vehicle-treated sham group; ##P < 0.01 compared with the insulin-treated IRI group. &&P < 0.01, compared with the vehicle-treated IRI group. n = 6 per group. HPF means high power field. N=6 in each group.

Results

The Changes in the Blood Glucose Concentration Over Time After Treatment of Insulin and Glucose

To maximize insulin dose, all mice (n=30 in this experiment, n=6 for each dose) in this study were supplied with 10% glucose drinking water. Insulin was injected subcutaneously twice at an interval time of 12 h. Both 0.1 U and 0.15 U per mouse led to hypoglycemia defined as a blood glucose concentration below 4 mmol/L (Figure 1). The doses of 0.025 and 0.05 units per mouse did not cause hypoglycemia (Figure 1). To induce insulin action in the kidneys, an insulin dose of 0.05 units per mouse was chosen to determine whether preconditioning with insulin and glucose could protect the kidney from IRI.

Preconditioning with Insulin and Glucose for 24 h Protected the Kidney from IRI

The mice subjected to kidney IRI developed renal dysfunction, which manifested by significant increases in serum creatinine and BUN (Figure 2A and B). Preconditional insulin preserved the renal function against IRI. The histological studies revealed that the renal damage in IRI was characterized by tubular dilation, sloughing of tubular epithelial cells,

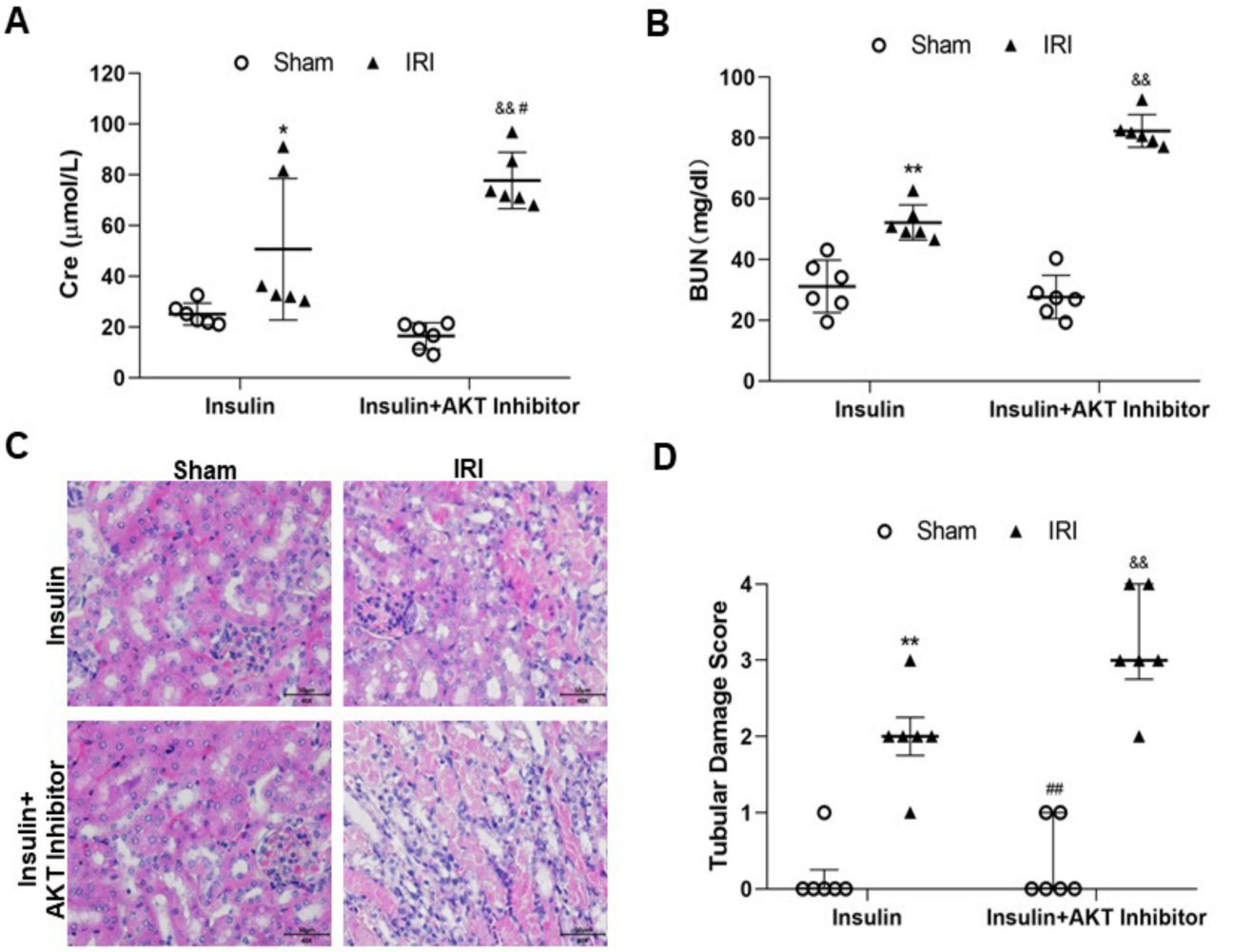

Figure 6 P-AKT inhibitor reversed the protection of insulin and glucose in the IRI kidney. (**A**) Summarized data showing the changes in serum Cre concentration of the four groups (Sham surgery with insulin injection, IRI surgery with insulin injection, Sham surgery with Insulin-and-AKT inhibitor injection, and IRI surgery with insulin-and-AKT inhibitor injection). *P < 0.05, compared with the insulin-treated sham group. $^{\&\&}$P < 0.01, compared with the insulin+AKT inhibitor-treated sham group. $^{\#}$P < 0.05, compared with the insulin-treated IRI group. (**B**) Summarized data showing the changes in serum BUN concentration in mice of the four groups. **P < 0.01, compared with the insulin-treated sham group. $^{\&\&}$P < 0.01, compared with the insulin-treated IRI group. (**C**) Representative renal H&E staining images of the four groups. (**D**) Summarized tubular damage score of the four groups. **P < 0.01, compared with the insulin-treated sham group. $^{\#\#}$P < 0.01, compared with the insulin-and-AKT inhibitor treated IRI group. $^{\&\&}$P < 0.01, compared with the insulin-treated IRI group. N=6 in each group.

cast formation, and loss of the brush border in the tubular epithelial cells (Figure 2C). The tubular damage score was used to evaluate the severity of renal damage. Preconditional Insulin reduced IRI damage as demonstrated by a decrease in the tubular damage score (Figure 2D). These results suggested that preconditional insulin protected the kidney from IRI.

Insulin and Glucose Activate AKT and Reduce BAX and Caspase-3 Expression

Immunofluorescence analysis for phosphorylated AKT (P-AKT) revealed that insulin activate AKT around the nuclei of tubular epithelial cells (Figure 3A). These data indicate that P-AKT is transferred from the cytoplasm to the nuclei after renal IRI. Bax—a central component of apoptotic signaling—was highly expressed in the nucleus of tubular epithelial cells in the kidney after IRI (Figure 3B). Caspase-3, a critical executioner of apoptosis, was detected in the nucleus of tubular epithelial cells in the kidney after IRI (Figure 3C). Statistical analysis indicated that insulin-induced AKT activation could attenuate the expression of Bax and caspase-3 in the kidney after IRI (Figure 3D–F).

Western blot analysis revealed that insulin could activate AKT by P-AKT in the kidney (Figure 4A). The activation of AKT (Figure 4A and B) decreased the expression of Bax and caspase-3 after IRI in the kidney (Figure 4A, C, and D). The Western blot results were consistent with the immunofluorescence results. These results suggest that preconditional insulin may protect the kidney from IRI through the inhibition of the apoptotic signaling pathway.

Preconditioning with Insulin and Glucose Decreased IRI-Induced Tubular Apoptosis

To further confirm the anti-apoptosis effect of insulin, TUNEL assay was performed to detect the apoptosis in the kidney (Figure 5A). Insulin preconditional significantly reduced apoptotic cell death in the kidney caused by IRI (Figure 5B).

The Protective Effect of Insulin and Glucose Was Reversed by an AKT Inhibitor

Based on the aforementioned results, preconditional insulin protects the kidney from IRI through the activation of AKT, which subsequently attenuates renal apoptosis. To further confirm the critical role of AKT in insulin-induced renal protection, an AKT inhibitor was used to assess the effect of insulin on the IRI kidney. The AKT inhibitor exacerbated renal dysfunction and augmented the IRI of the kidney in the presence of insulin (Figure 6A–D). The number of apoptotic cells increased when the AKT inhibitor was administered with insulin (Figure 7A and B). Western blot analysis

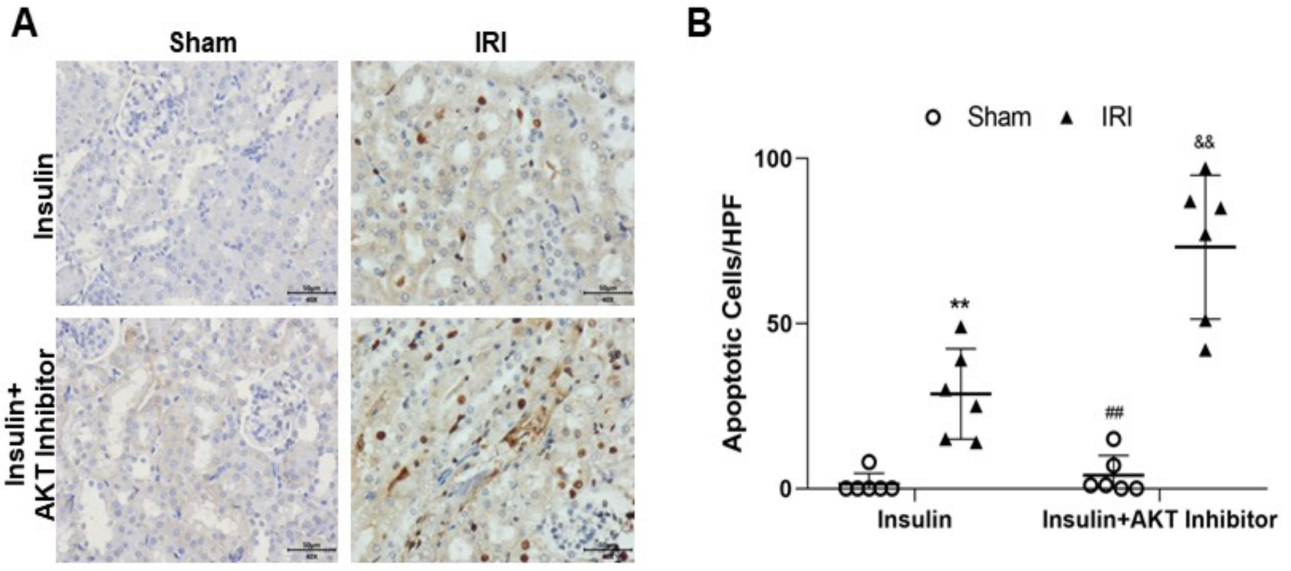

Figure 7 P-AKT inhibitor reversed the insulin-induced improvement in apoptosis in the IRI kidney. (**A**) Representative results of apoptosis in the kidneys with apoptotic cells (brown) counterstaining with hematoxylin (blue) (original magnification is 400×) of the four groups (Sham surgery with insulin injection, IRI surgery with insulin injection, Sham surgery with Insulin-and-AKT inhibitor injection, and IRI surgery with insulin-and-AKT inhibitor injection). (**B**) The apoptotic cells were quantitatively analyzed in the kidneys. $^{**}P < 0.01$ compared with the mice treated with Sham surgery and insulin injection; $^{\#\#}P < 0.01$ compared with the mice treated with IRI surgery and insulin and AKT inhibitor injection. $^{\&\&}P < 0.01$, compared with the mice treated with IRI surgery and insulin injection. n = 6 per group. HPF means high power field. N=6 in each group.

revealed that the AKT inhibitor blocked insulin-induced AKT activation (Figure 8A–D). In the presence of insulin, an AKT inhibitor also significantly increased Bax/caspase-3 expression and exacerbated renal apoptosis in IRI kidneys. These results suggest that insulin-induced renal protection depends on AKT activation and subsequent attenuation of apoptosis. N=6 in each group.

Extension of Precondition Duration Failed to Improve Continuously the Protective Effect on Renal IRI

Since preconditional insulin protects the kidney from IRI, an extension of the preconditional time may time-dependently improve renal protection. To confirm the effect of preconditional time, we compared the renal protective effects of insulin preconditional for 1, 3, and 6 days. However, there were no differences in creatinine, BUN, and tubular damage score between 1-, 3-, and 6-day insulin precondition (Figure 9A–D). There were no differences in P-AKT/Bax/caspase-3

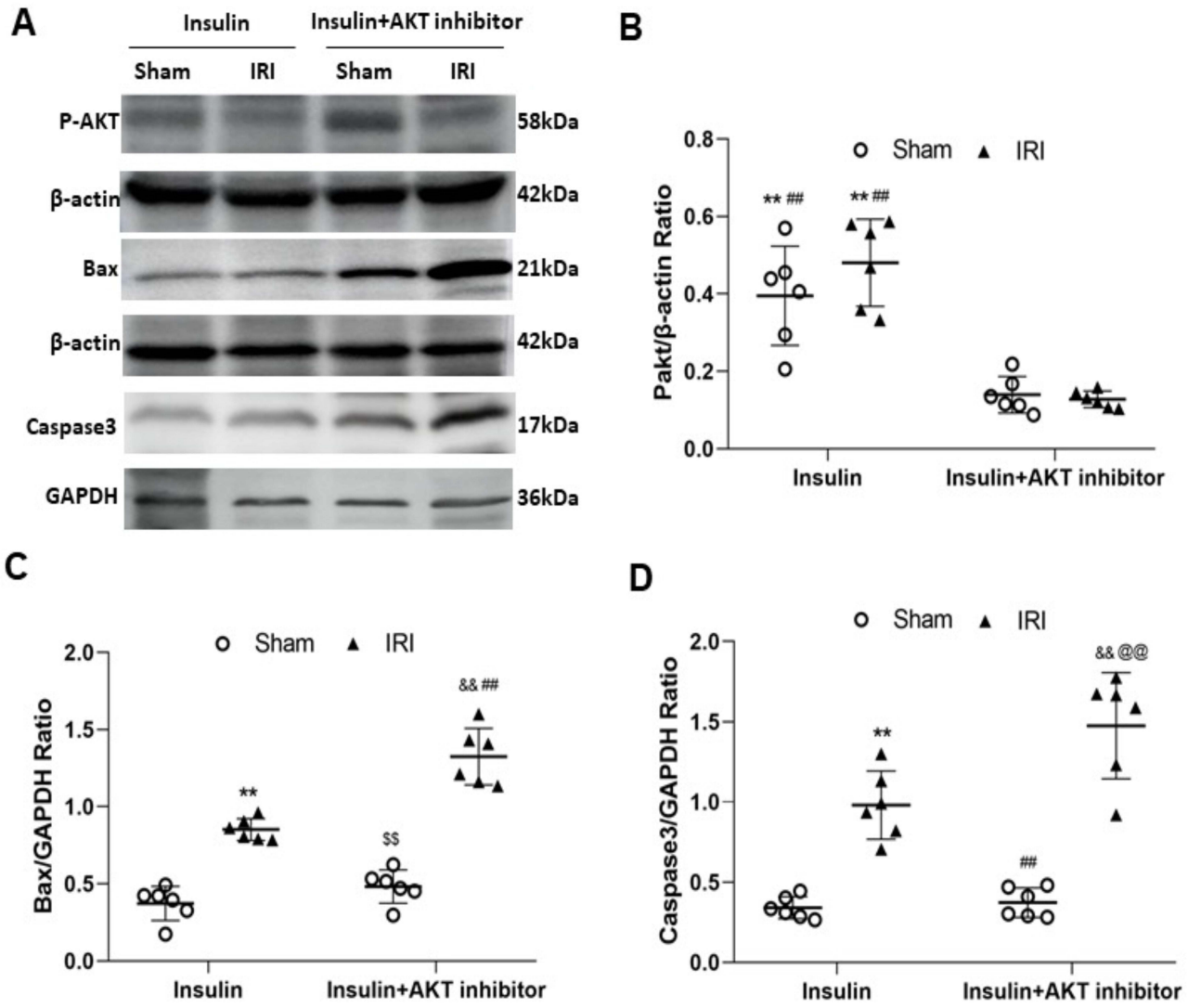

Figure 8 Western blot results of P-AKT, Bax, and caspase-3 expression in the kidney after IRI in the presence of insulin and the AKT inhibitor. (**A**) Representative results of P-AKT, Bax, and caspase-3 expression in Western blot results of the four groups (Sham surgery with insulin injection, IRI surgery with insulin injection, Sham surgery with Insulin-and-AKT inhibitor injection, and IRI surgery with Insulin-and-AKT inhibitor injection). (**B**) Quantitative analysis of P-AKT expression in the kidneys. **P < 0.01, compared with the sham group treated with insulin and AKT inhibitor. $^{\#\#}$P < 0.01, compared with the IRI group treated with insulin and the AKT inhibitor. (**C**) Quantitative analysis of Bax expression in the kidneys. **P < 0.01, compared with the insulin-treated sham group. $^{\$\$}$P<0.01, compared with the insulin-treated IRI group. $^{\&\&}$P< 0.01, compared with the insulin+AKT inhibitor-treated Sham group. $^{\#\#}$P < 0.01, compared with the IRI group treated with insulin and AKT inhibitor. (**D**) Quantitative analysis of cleaved caspase-3 expression in the kidneys. **P < 0.01, compared with the insulin-treated sham group. $^{\#\#}$P < 0.01, compared with the IRI group treated with insulin only. $^{\&\&}$P < 0.01, compared with the insulin+AKT inhibitor-treated Sham group. $^{@@}$P<0.01, compared to the insulin-treated IRI group. N=6 in each group.

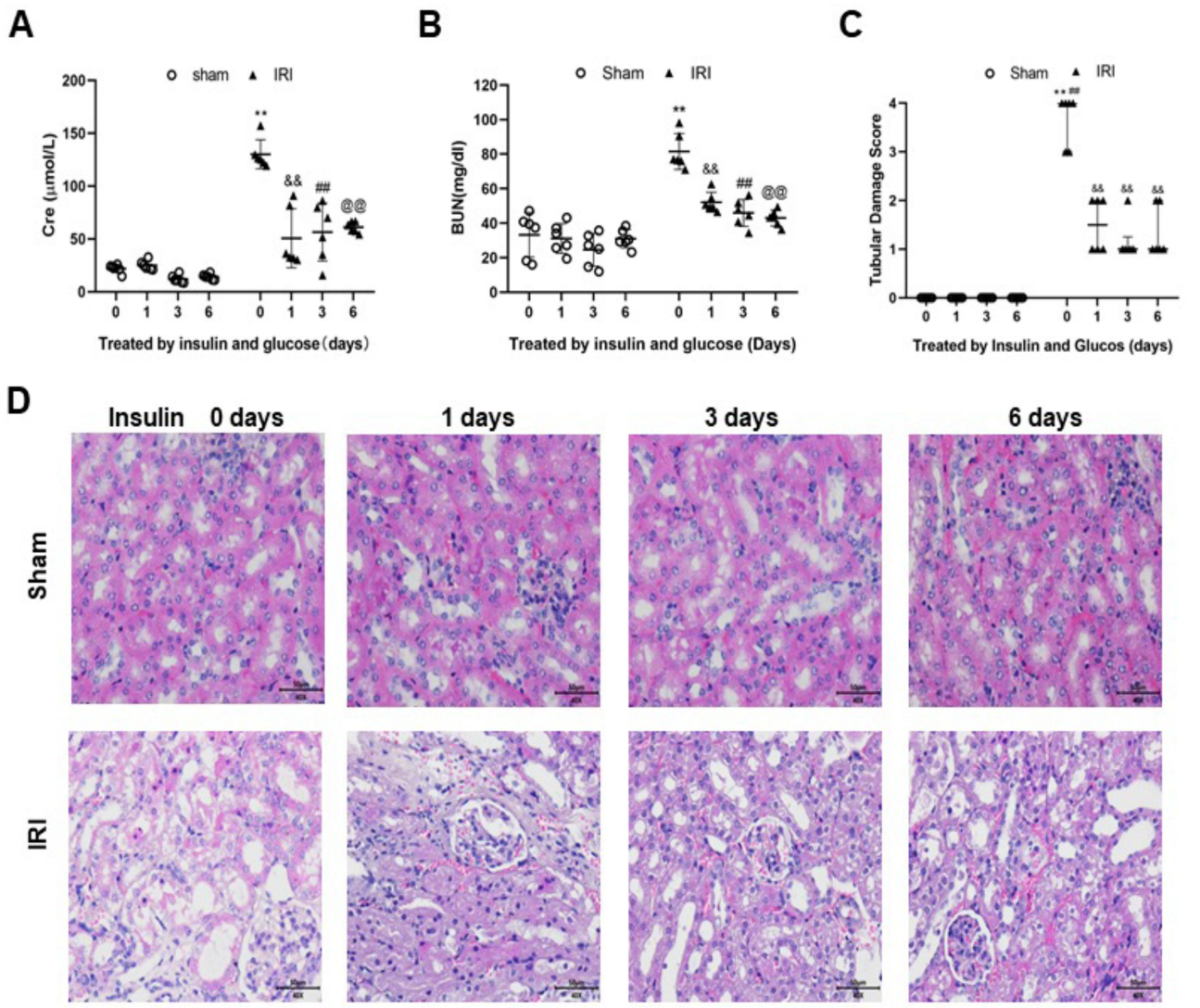

Figure 9 The mice were pretreated with insulin for varying time periods (1 d, 3 d, and 6 d, Twice a day, 0.5 U per time). The mice received the same treatment without renal pedicle clamping was used as a sham surgery. (**A**) The effects of insulin on Cre changes in mice with/without IRI. **P < 0.01, compared with the insulin-treated sham group (0, 1, 3, 6 days). $^{\&\&}$P < 0.01, compared with the IRI group treated with insulin for 0 day. $^{\#\#}$P < 0.01, compared with the IRI group treated with insulin for 0 day. $^{@@}$P < 0.01, compared with the IRI group treated with insulin for 0 day. (**B**) The effects of insulin on BUN changes in mice with/without IRI. **P < 0.01, compared with the insulin-treated sham group (0, 1, 3, 6 days). $^{\&\&}$P < 0.01, compared with the IRI group treated with insulin for 0 day. $^{\#\#}$P < 0.01, compared with the IRI group treated with insulin for 0 day. $^{@@}$P < 0.01, compared with the IRI group treated with insulin for 0 day. (**C**) Tubular damage score in C57/B6 mice with or without ischemia /reperfusion. **P < 0.01, compared with the insulin-treated sham group (0, 1, 3, 6 days). $^{\#\#}$P < 0.01, compared with the IRI group treated with insulin for 1, 3, 6 day. $^{\&\&}$P < 0.01, compared with the insulin-treated sham group for 0, 1, 3, and 6 days. (**D**) Representative images of HE staining after insulin injection for 0, 1, 3, and 6 days. The mice were subjected to sham or IRI surgery. N=6 in each group.

expression after 1-, 3-, or 6-day insulin precondition (Figure 10A–D). TUNEL assay revealed no differences in apoptotic cell death in the kidney after 1-, 3-, or 6-days insulin precondition (Figures 11 and 12). The results indicated that a short-term insulin precondition (24 h) is sufficient to induce renal protection against IRI.

Discussion

This study demonstrated that a 24-h preconditioning with insulin and glucose efficiently prevents kidney IRI of mice. First, an ideal dose of insulin per mouse for precondition was chosen to avoid hypoglycemia (below 4 mmol/L) through the detection of blood glucose. Preconditioning with insulin and glucose protected the kidney through the reduction of tubular apoptosis via the P-AKT/Bax/caspase-3 signal pathway. The signaling of insulin-induced renal protection was

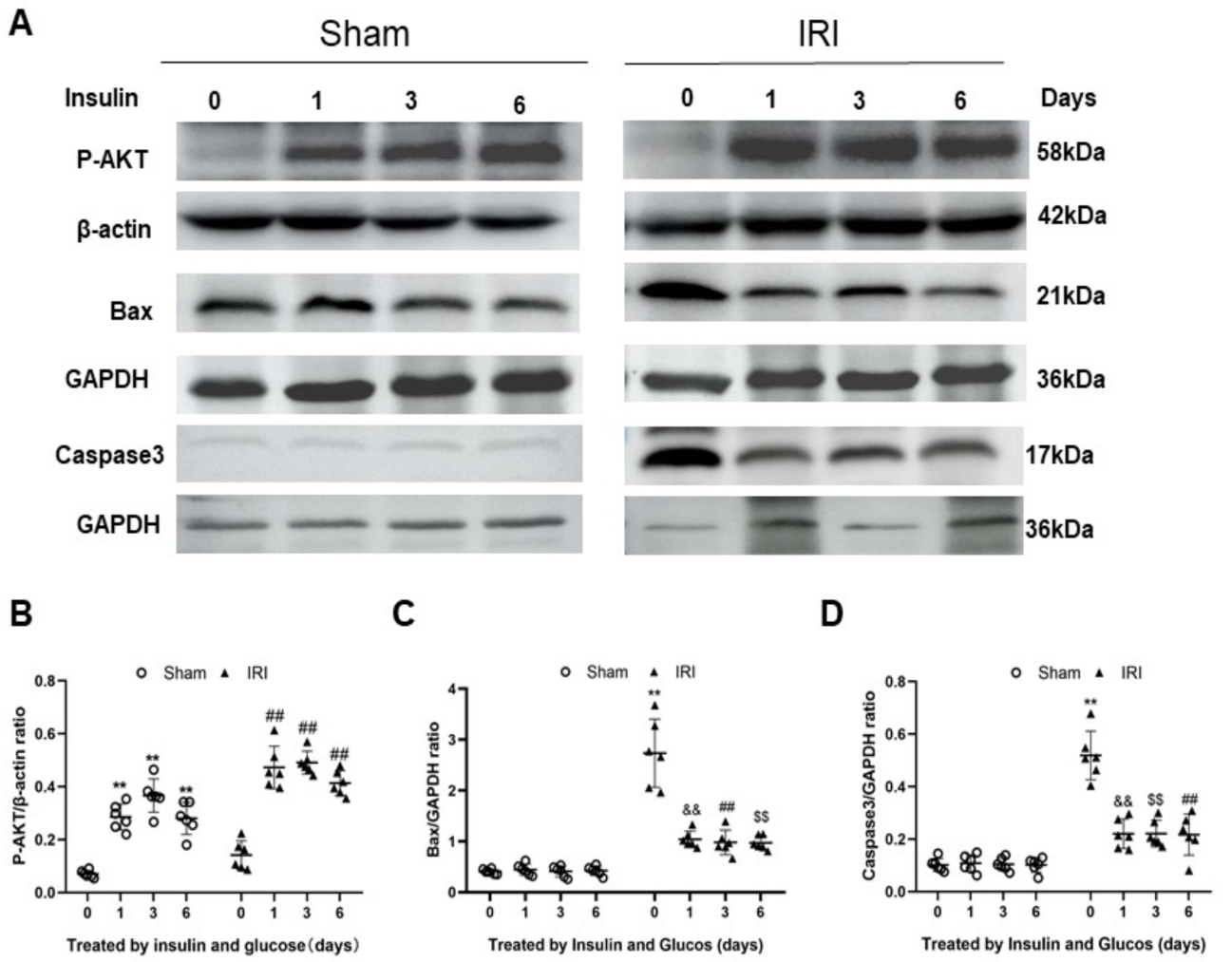

Figure 10 The P-AKT, Bax, and caspase-3 expression in the kidneys after different preconditional durations of insulin and glucose. (**A**) Representative results of P-AKT, Bax, and caspase-3 expression in Western blot in insulin-treated (insulin injection for 0, 1, 3, and 6 days) mice that were subjected to sham or IRI kidney surgery. (**B**) Quantitative analysis of P-AKT expression in the Western blot. **P < 0.01, compared with the sham group treated with insulin for 0 day. ##P < 0.01, compared with the IRI group treated insulin for 0 day. (**C**) Quantitative analysis of Bax expression in the Western blot. **P < 0.01, compared with the insulin-treated sham group (0, 1, 3, 6 days). &&P < 0.01, compared with the IRI group treated with insulin for 0 day. ##P < 0.01, compared with the IRI group treated with insulin for 0 day. $$P < 0.01, compared with the IRI group treated with insulin for 0 day. (**D**) Quantitative analysis of cleaved caspase-3 expression in the Western blot. **P < 0.01, compared the sham group treated with insulin for 0, 1, 3, or 6 days. &&P < 0.01, compared with the IRI group treated with insulin for 0 day. $$P < 0.01, compared with the IRI group treated with insulin for 0 day. ##P < 0.01, compared with the IRI group treated with insulin for 0 day. N=6 in each group.

further confirmed using an AKT inhibitor. Finally, the effects of preconditional duration were investigated between 1-, 3-, and 6-day treatments. The 24-h precondition was found to have similar renal protection as either 3- or 6-day treatment.

Although preconditional insulin was found renal protection in DM mice, no study was performed in normal mice.[22] Intensive insulin therapy displayed controversial effects in renal protection in clinics but caused a definitive complication, hypoglycemia.[17,23,24] Fortunately, this study provided an alternative method for utilizing the renal-protective benefits of insulin by administering insulin and glucose together before renal IRI. This study systemically presented a practicable method to protect the kidney against IRI by choosing insulin doses, determining the mechanism, and optimizing treatment duration.

To enhance the effect of insulin on the kidney, we administered 10% glucose drinking water to the mice to maximize the insulin dose and avoid hypoglycemia. In this study, hypoglycemia in mice was defined as below 4 mmol/L (72 mg/dL) to minimize any adverse effect of insulin.[25] Depressive-like behaviors or neurogenic adverse effects occurred at blood glucose concentrations below 50–60 mg/dL (2.8–3.3mmol/L).[26] However, epinephrine secretion usually occurred at blood glucose concentrations below 70 mg/dL (3.9mmol/L). Therefore, the definition of hypoglycemia was chosen as the blood glucose concentration below 4 mmol/L to adjust the insulin dose in this study.

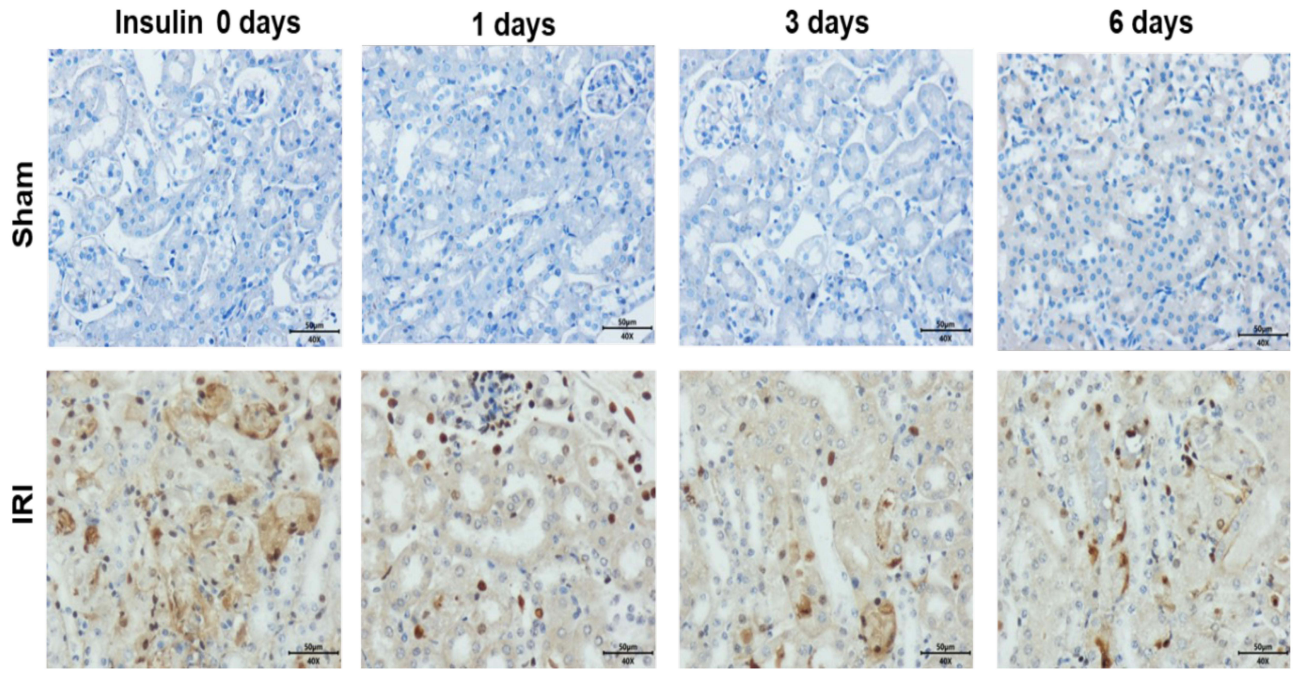

Figure 11 Representative images of TUNEL staining of the kidneys. The apoptotic cells (brown) counterstaining with hematoxylin (blue) (magnification is 400×). Mice were preconditioned to insulin for different periods (1day 3days or 6days, twice a day, 0.5U each time). N=6 in each group.

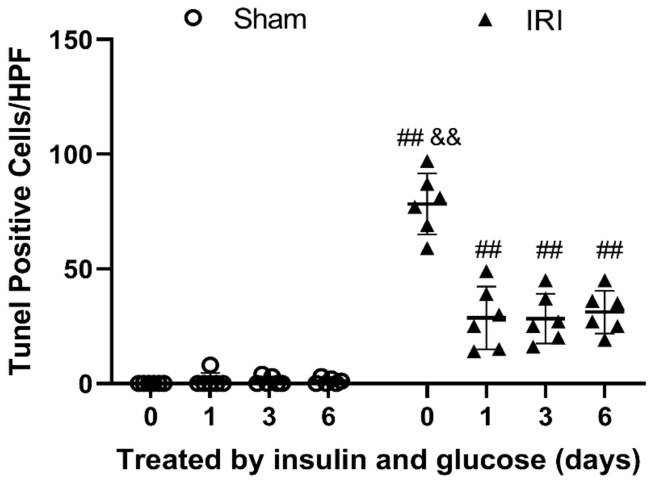

Figure 12 The apoptotic cells were quantitatively analyzed in the kidneys after treated with insulin for 0, 1, 3, and 6 days. $^{\#\#}P < 0.01$ compared to sham group treated with insulin and glucose for 0, 1, 3, and 6 days respectively. $^{\&\&}P < 0.01$, compared to IRI group treated with insulin and glucose for 1, 3, and 6 days respectively. n=6 per group. HPF means high power field (400 (**X**)).

In this study, we discovered that preconditioning with insulin and glucose protected the kidney from subsequent IRI. To elucidate the mechanism of the renal protective effect of insulin, we investigated the key component, P-AKT, in insulin signaling in this study.[27] Insulin was previously found to decrease caspase-3 activity in human renal tubular epithelial cells through PI3K/AKT signaling.[6] In an animal experiment, insulin was reported to protect the kidney after IRI through the reduction of tubular apoptosis.[10] Therefore, the signaling pathway of P-AKT/Bax/caspase-3 involving insulin and apoptosis was chosen to evaluate the renal protective effect of insulin treatment before IRI. Immunofluorescence results indicated that insulin increased P-AKT expression around the nucleus, which subsequently enters into the nucleus in tubular cells after IRI. However, it was reported that AKTs cannot translocate from cytoplasma

to nucleus after stimulation. Actually, AKT has three isoforms AKT1, AKT2, and AKT3. AKT1, AKT2, and AKT3 locate respectively in cytoplama, mitochondrial, and nuclear membrane. AKT2 was reported involved in glucose metabolism and inhibiting apoptosis.[19,28] These three isoforms were not differentiated in this study. The insulin-activated P-AKT may inhibit the apoptosis of tubular epithelial cells through a decrease in the expression of Bax and cleaved caspase-3 in the nucleus. The Western blot results were consistent with the immunofluorescence results in the expression of P-AKT/Bax/caspase-3 signaling. The role of P-AKT/Bax/caspase-3 signaling in insulin-induced renal protection was confirmed using an AKT inhibitor in this study.

Preconditioning with insulin and glucose before IRI is easy to practice in clinical conditions. To fine-tune the preconditioning with insulin and glucose, we investigated whether extending the preconditional time can continuously boost insulin-induced renal protection. Surprisingly, there were no differences in renal protection between 0-, 1-, 3-, and 6-day precondition. It was concluded that a short-term 24-h precondition is enough to induce renal protection. This finding facilitates the preconditioning with insulin and glucose in clinical conditions where IRI in the kidney usually occurs, such as heart and large vessel surgeries. A further clinical trial is warranted to confirm the renal protective effect induced by insulin and glucose, given that the precondition is acceptable and practicable without any damage.

Conclusion

A short-term preconditioning with insulin and glucose protected the kidney from IRI through activation of p-AKT and subsequent reduction of the BAX-caspase-3-induced apoptosis. The short-term precondition provides a practicable method for protecting the kidney from predicable IRI.

Data Sharing Statement

The data and material are available from the corresponding author if a proper quest is received.

Ethics Approval and Consent to Participate

This study was approved by the ethics committee of Zhengzhou Central Hospital of Zhengzhou University.

Acknowledgments

This paper is available as a preprint on Research Square at https://www.researchsquare.com/article/rs-2633530/v1.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

This study was supported by the projection of priority and extensive application in research in Henan province (222102310019) and the projection of cooperative construction from department of science and technology of Henan province (SBGJ202102209).

Disclosure

The authors declare no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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