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Intravenous infusion of ulinastatin attenuates acute kidney injury after cold ischemia/reperfusion

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Abstract

Background Administration of ulinastatin was proved to protect many organs from ischemia/reperfusion (I/R) induced injury, yet its protective effects on renal I/R injury under cold condition and mechanism still remain unclear.

Aims In the present study, the protective effects of ulinastatin on renal cold I/R injury as well as its mechanism were investigated.

Methods and results Renal cold I/R model was constructed via cross-clamping of left renal artery and vein at 4 °C. The ulinastatin was administrated and multi-methods were performed to evaluate the protective effects. The results showed that ulinastatin could mitigate the renal cold I/R injury. In addition, the attenuated kidney cold I/R injury by ulinastatin was also accompanied with its regulating capability of the microenvironment, such as decreased acute inflammatory response, oxidative stress damage and apoptosis, as well as attenuation of vasculature levels decrease, as evidence by reduced TNF-α, IL-6 mRNA expression, MDA levels and apoptosis, higher levels of SOD activity and CD31/α-SMA expression.

Conclusion The present study suggested that ulinastatin might be clinically useful in reducing preservation injury induced by cold I/R during renal transplantation surgery.

Keywords Ulinastatin · Cold I/R · Renal injury · Protective effects

Introduction

Kidney transplantation remains the only definitive treatment for end-stage renal disease so far, and the function preserving of the donor kidney grafts is vital for effective transplantation [1, 2]. Despite the fact that the hypothermic pulsatile machine perfusion used during kidney transplantation surgery is effective in attenuating the preservation injury for high surgical success rate following the surgery [3], the kidneys from all donor types are sensitive to ischemia and the reperfusion injury is still inevitable [4–6]. The acute kidney injury after cold ischemia/reperfusion might lead to a high incidence of delayed graft function (DGF) after kidney transplantation [7, 8].

It is therefore necessary to mitigate preservation injury in most complicated renal operations by administration of some protective drugs [6]. However, the pharmacological renal protection strategies for kidney cold ischemia/reperfusion still remain the challenge to date. Ulinastatin, a urinary trypsin inhibitor (UTI) that could inhibit various inflammatory proteases such as chymotrypsin and neutrophil elastase, was proved to have protective effects on many organs [9, 10]. Ulinastatin is a glycoprotein with molecular weight of 25–25 kDa. It could be purified from healthy human urine or synthetically produced, and highly purified ulinastatin has been clinically used for the treatment of acute or chronic pancreatitis, severe infection, Stevens–Johnson syndrome, etc. [11, 12]. Despite the fact that the maximum recommended daily dose of ulinastatin is 3×10^5 U (listed in the package of ulinastatin drug), the clinically used doses of ulinastatin were normally allowed to achieve therapeutic concentrations for severe acute diseases with higher concentration [12–14].

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Recent studies suggested that the administration of ulinastatin could also protect many organs from ischemia/reperfusion-induced injury and has been widely used for postoperative organs protection and pancreatitis, etc. [15–19]. Although previously Chen had investigated the protective effects of ulinastatin on renal ischemia–reperfusion injury under normal condition, the underlying mechanism still remains unclear [20]. Especially, the effects of ulinastatin on acute renal injury after cold ischemia/reperfusion as well as its underlying mechanism were rarely reported.

To address this, the present study aims to investigate whether ulinastatin has a protective role in cold preservation of kidney, its regulation effects on the local microenvironment, and explore the possible protective mechanism of ulinastatin on acute kidney injury after cold ischemia/reperfusion. The renal cold I/R injury indicators serum creatinine and urea nitrogen were analyzed. The mRNA expression levels pro-inflammatory cytokines TNF-α and IL-6, oxidative stress indicators MDA and SOD, as well as cellular apoptosis and its related proteins were examined. The microvascular pathological changes of injury renal were detected via CD31 and α-SMA detection as well.

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing 250–300 g were bought from the Experimental Animal Center, Academy of Military Medical Science (Beijing, China). All the animals were kept in a standard animal laboratory with free activity and free access to water and chow. The surgery operations were performed under clean conditions and the procedures used for the study were approved by the local institution of Animal Experimental Ethics Committee of Experimental Animal Center, Academy of Military Medical Science (Beijing, China).

Renal cold I/R model

Animals were prepared and underwent surgery using the method as described [21]. Briefly, the rats were fasted for 12 h before surgery and anesthetized by intraperitoneal administration of Chloral Hydrate (60 mg/kg). Two kidneys of a rat were exposed and the right kidney was removed through a midline laparotomy. After occluding the left renal artery and vein by the micro-clamps, a 0.7 mm × 19.0 mm needle was placed in the left renal artery and a 3 mm outflow rupture was also created to allow drainage of the perfusion solution. The left kidney was then perfused with 10 ml cold saline solution in 2–3 min at 4 °C. The cross-clamping of left renal artery and vein was then performed for 45 min, during which the needles in the artery and vein were removed and the rupture were sealed. At the end of the surgery, micro-clamps were removed away for recovery of the left renal blood flow.

Time-dependent effect of the renal cold I/R injury

In order to choose the appropriate time points for further ulinastatin protective effects investigation, we first evaluated the time-dependent effects of cold I/R on the renal injury. Briefly, the rat renal cold I/R model was first constructed. After that, the rats were killed at different time points (0–50 h) after surgery, and the abdominal aortic blood and kidneys samples were then obtained from the rats for serum creatinine and urea nitrogen analysis. 5 animals at each time points were applied.

Experimental groups

To investigate the protective effects of ulinastatin, the rats were randomized into three groups. For Sham group ($n = 8$), the rats were subjected to surgery without the I/R episode. For cold I/R group ($n = 8$), the rat renal cold I/R model was constructed as mentioned above. For cold I/R + ulinastatin group, the rats were first injected with ulinastatin (5 kU/kg) via carotid artery 30 min before rat renal cold I/R surgery, and the ulinastatin (5 kU/kg) was also administrated simultaneously at the end of I/R surgery as well. The rats were sacrificed 24 h after surgery for further evaluations. Blood and renal samples were collected for serum creatinine and urea nitrogen analysis. Parts of each kidney was then used to extract total mRNA and protein for molecular experiments. The remaining tissue was then fixed in 10% buffered formalin for histological analysis.

Malondialdehyde (MDA) and superoxide dismutase (SOD) measurement

To evaluate the antioxidant effect of the ulinastatin on I/R injury, the renal tissue from Sham group, Cold I/R group and cold I/R + ulinastatin group was damaged to obtain the tissue homogenates. For MDA levels measurement in homogenates, thiobarbituric acid-reactive substances were measured using a Lipid Peroxidation MDA Assay Kit (Beyotime, China). MDA values are calculated according to the manufacturer's instructions and expressed as mM. For enzyme activities superoxide dismutase (SOD) measurement, the Total Superoxide Dismutase Assay Kit with WST-1 (Beyotime, China) was used according to the manufacturer's instructions.

qRT-PCR analysis

The total mRNA of renal tissue was extracted using Trizol reagent according to manufacturer instructions (Invitrogen). The first-strand cDNA was synthesized according to the protocol the use of random primer and PrimeScript RT reagent Kit (Takara, China). Quantitative real-time PCR (qRT-PCR) was analyzed with a total reaction volume of 10 μL and a total of 40 cycles. The reaction was performed using a Bio-Rad CFX (Bio-Rad, USA) and SYBR Select Master Mix (Thermo scientific, Logan, USA). IL-6 and TNF-a were analyzed and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as housekeeping gene. The level of target molecule mRNA was expressed via being normalized to the housekeeping gene GADPH. Primers used for the amplification using the following primers: 5′-GCCCT TCAGGA ACAGC TATGA-3′ (forward) and 5′-TGTCA ACAAC ATCAG TCCCAA GA-3′ (reverse) for rat IL-6, 5′-AAATG GGCTC CCTCT CATCAG TTC-3′ (forward) and 5′-TCTGC TTGGT GGTTT GCTAC GAC-3′ (reverse) for rat TNF-α, 5′-GACAT GCCGC CTGGA GAAAC-3′ (forward) and 5′-AGCCC AGGAT GCCCT TTAGT-3′ (reverse) for rat GAPDH. All the primers were obtained from Sangon, China. At least three independent experiments were performed for each group.

Western blotting analysis

The renal tissue was lysed with Laemmli Sample Buffer (Bio-Rad) and the protein concentration was quantified using a BCA protein assay Kit (Solarbio, China). Equal amounts (60 μg) of extracted proteins were loaded onto 12% polyacrylamide gel and separated by electrophoresis. The proteins were then transferred onto polyvinylidene difluoride (PVDF) (Roche) membrane. After blocking with 5% defatted milk for 1 h, the samples were then incubated with primary antibodies at 4 °C overnight and with appropriate horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. The signals of protein bands were detected by enhanced chemiluminescence reagent (Applygen). Using Molecular Imager Versa Doc MP 4000 System (Bio-Rad). Band intensity was normalized to β-actin. The following antibodies were used for immune-blotting: cleaved Caspase-3 (1:1000, cleaved csp3, number 9661S), cleaved Caspase-7 (1:1000, cleaved csp7, number 8438T), Bcl-xl (1:1000, number 2764S), Bcl-2 (1:1000, number 3498S), CD31 (1:1000, number 3528S), vWF (1:1000, number 65707S) and β-actin (1:8000, number 4970S) were obtained from cell Signaling Technology (USA).

Histology analysis

The fixed samples were dehydrated with alcohol of graded concentrations and embedded with paraffin. 3–5 μm sections were prepared, and stained with hematoxylin and eosin (H&E), TUNEL and CD31, respectively. Briefly, the paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol. The sections were then incubated with proteinase K for 20 min at 37 °C for permeabilization. After that, the endogenous peroxidase activity was quenched by 3% hydrogen peroxide treatment for 10 min. For TUNEL staining (Beyotime, Beijing, China), biotin-labeled liquid, biotinylated nucleotide and TdT enzyme were incubated for 1 h at 37 °C under darkness. The sections were then treated with streptavidin–HRP for 30 min at room temperature and stained with staining with 3,3′-diaminobenzidine (DAB) for 5 min. The nucleuses were stained with hematoxylin. For CD31 staining, the sections were incubated with mouse CD31 primary antibody overnight at 4 °C, and followed by incubation with a Cy3-conjugated second antibody (Abcam, USA). The stained slices were observed with Olympus IX71 inverted microscope (Japan). Images from 2–3 random fields in each group were obtained and 5–7 animals from each group were included. The percentage of apoptotic cells from each group was calculated from the TUNEL staining images, and the micro-vascular levels were also quantified according to the CD31 staining images.

Statistical analysis

Data are expressed as mean ± SD. Differences between the groups were evaluated with the use of one-way analysis of variance (ANOVA). *P* values of $p < 0.05$ were set to be statistically significant.

Results

Protective effect of ulinastatin on renal cold I/R injury

In order to evaluate the time-dependent effect of the renal cold I/R injury, the serum creatinine and urea nitrogen were analyzed at different time points after surgery. As shown in Fig. 1, the results of both serum creatinine (Cr) and urea nitrogen (BUN) indicated that cold ischemia–reperfusion induced a time-dependent function injury of the renal within 24 h after the operation. What's more, the BUN and Cr reached the highest levels at the time point of 24 h. However, both BUN and Cr were observed with a slightly downward trend after 24 h. Therefore, the time points of 24 h after surgery were used to for the following renal functional study.

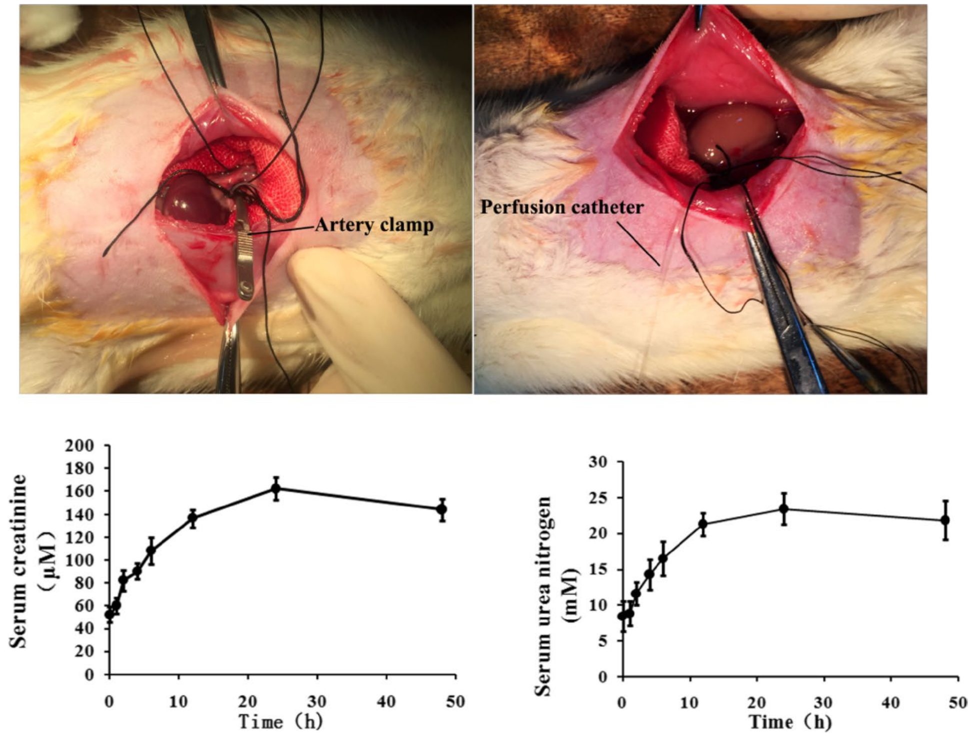

Fig. 1 The construction of the renal cold ischemia/reperfusion injury, and its time-dependent effect on the serum creatinine and urea nitrogen levels ($n = 5$)

As shown in Fig. 2, cold I/R injury lead to significant increase of the BUN and Cr levels as compared to sham operated control group. However, both BUN and Cr levels were markedly decreased 24 h after operation via the administration of ulinastatin in cold I/R + ulinastatin group, suggesting that ulinastatin could suppress cold I/R-induced renal injury.

Inhibition of ulinastatin on TNF-α and IL-6 production

In order to determine the possible anti-inflammation of ulinastatin, the mRNA expression levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) that are autocrine contributors to renal dysfunction, necrosis as well as apoptosis in I/R injury, were therefore examined using qRT-PCR. As shown in Fig. 3, cold I/R injury resulted in significant increase of the TNF-α and IL-6 mRNA levels as compared to control group. After ulinastatin treatment, the increase of TNF-α and IL-6 mRNA levels by cold I/R injury were partly attenuated, which indicated that the administration of ulinastatin could restore the increase of pro-inflammatory cytokines expression levels.

Suppression effect of ulinastatin on oxidative status in renal

To investigate whether ulinastatin could attenuate the oxidative stress during cold I/R injury, renal MDA levels and SOD activities were assessed. MDA is one of the most mutagenic

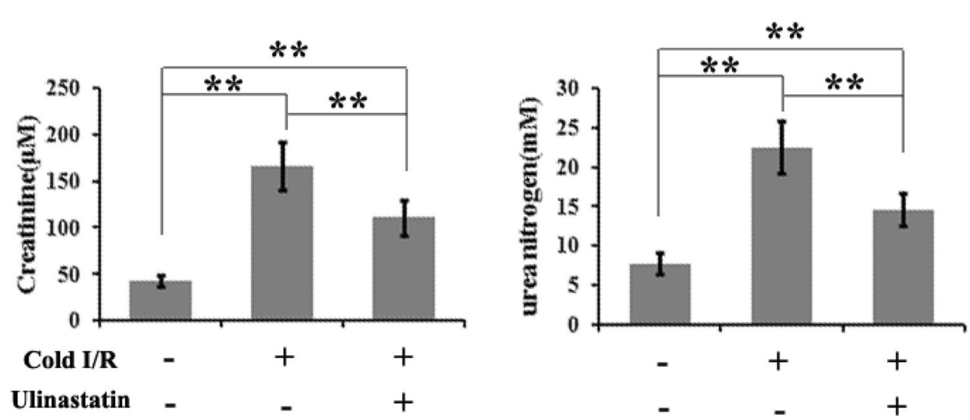

Fig. 2 The protective effects of ulinastatin on renal cold I/R injury as evaluated by serum creatinine and urea nitrogen levels, *$p < 0.05$, **$p < 0.01$

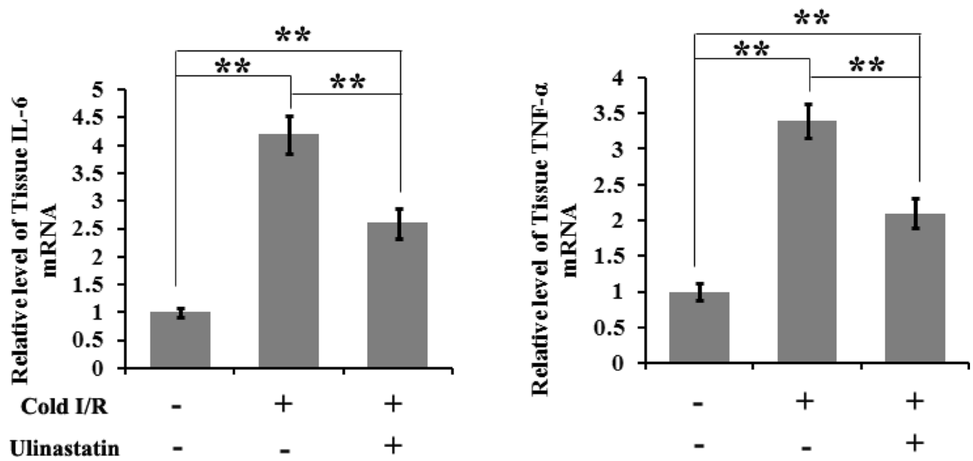

Fig. 3 The expression levels of the IL-6 and TNF-α in renal, *$p<0.05$, **$p<0.01$

product of lipid peroxidation, which makes it been widely used as a convenient biomarker for lipid peroxidation for evaluation of oxidative stress levels [22, 23]. The superoxide dismutases (SOD), which dismutate superoxide to hydrogen peroxide, are a natural defense against oxidative injury and an indicator for oxidative injury [24]. As shown in Fig. 4, MDA level was increased and the SOD activity was decreased in cold I/R injury group as compared with control group ($p<0.01$). While in cold I/R + ulinastatin group, the ulinastatin treatment significantly decreased renal MDA concentrations and increased SOD activity level ($p<0.01$) as compared to cold I/R group. These data suggested that renal-protective effect offered by ulinastatin might be associated with its antioxidant effect.

Protective effect of ulinastatin against renal histological injury and apoptosis

As shown in Fig. 5, as compared with Sham group, the cold I/R group were observed with much more morphologic abnormalities including cell necrosis of the proximal convoluted tubule, tubular lumen obstruction and cytoplasmic vacuolization. However, the morphologic abnormalities in cold I/R + ulinastatin group were partly restored. TUNEL staining results confirmed that cold I/R injury induced significant increase of TUNEL-positive cell, but it was significantly decreased in cold I/R + ulinastatin group via ulinastatin treatment. To further investigate the effect of ulinastatin on renal apoptosis, the apoptosis-related proteins production was evaluated using Western blotting. As shown in Fig. 5, the Western blotting results showed that the cleaved caspase-3, cleaved caspase-7 and Bcl-xl levels of renal were higher, and the Bcl-2 level of renal was lower in cold I/R group ($p<0.01$) than that of Sham group. After treating with ulinastatin, the increase of cleaved caspase-3, cleaved caspase-7 and Bcl-xl levels, and the decrease of Bcl-2 level were attenuated in cold I/R + ulinastatin group. These results indicated that ulinastatin could protect the renal from cold I/R injury through decrease of the anti-apoptotic effects, which was associated with apoptosis-related proteins, such as cleaved caspase-3, cleaved caspase-7, Bcl-2 and Bcl-xl.

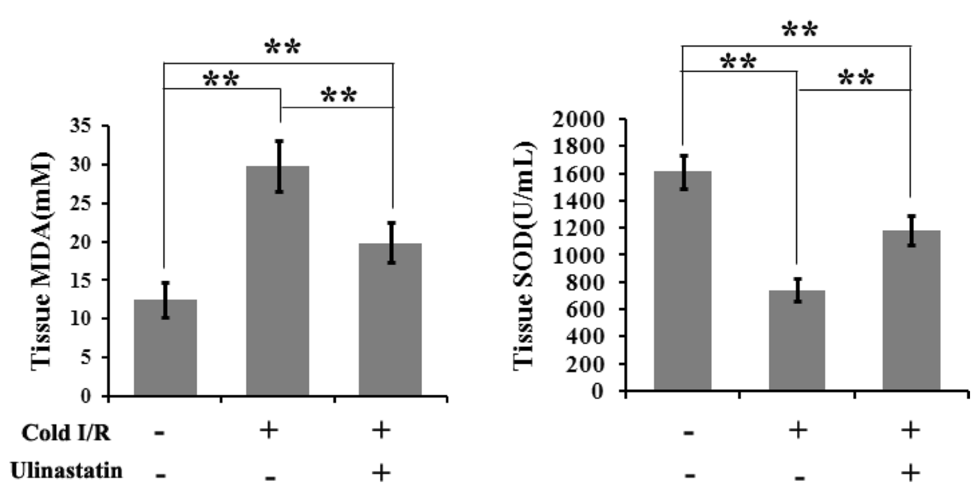

Fig. 4 The MDA levels and SOD activities of the renal, *$p<0.05$, **$p<0.01$

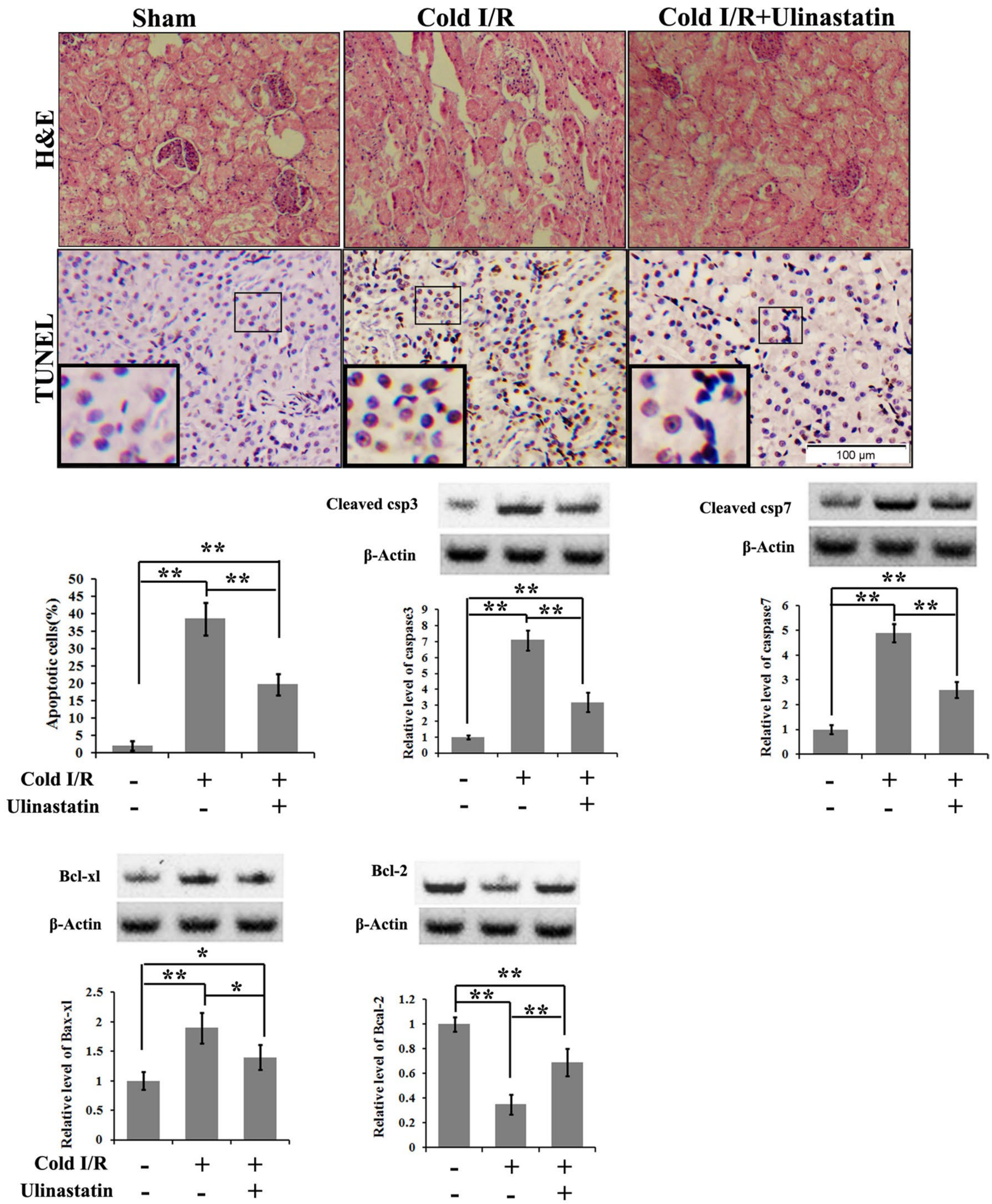

Fig. 5 H&E staining, TUNEL staining and its quantitative analysis of apoptotic cells percentage accordingly; as well as the Western blotting results of the cleaved caspase-3, cleaved caspase-7 Bcl-xl, Bcl-2 and the quantitative analysis results, $*p<0.05$, $**p<0.01$

Protective effect of ulinastatin on micro-vascular pathological changes

In order to investigate whether ulinastatin affect the micro-vascular pathological changes of injury renal by cold I/R, the expression levels of CD31 and α-SMA were detected by immunofluorescence staining and Western blotting, respectively. As shown in Fig. 6a, the CD31 staining results showed that the cold I/R injury decrease the expression level of CD31 as compared with Sham group, and the cold I/R + ulinastatin group was observed with higher levels of CD31 expression compared to cold I/R group. The Western blotting results of both CD31 and α-SMA confirmed that cold I/R injury induced significant decrease of the

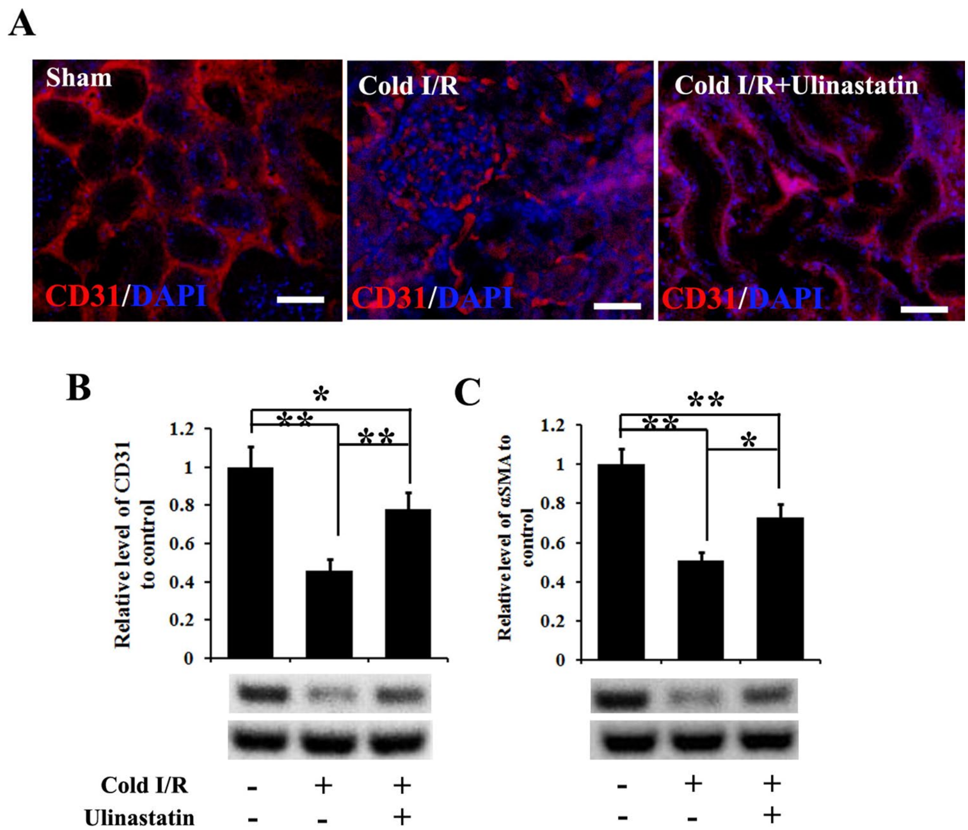

Fig. 6 CD31 staining (**a**), the Western blotting results of the CD31 (**b**) and α-SMA (**c**), as well as its quantitative analysis results, *$p<0.05$, **$p<0.01$. Scale bar = 50 µm

vasculature levels, which could be partly restored via ulinastatin administration in cold I/R + ulinastatin group.

Discussion

Perfusion preservation of kidneys is now widely used during the renal transplantation surgery, especially since the use of extended criteria donor (ECD) or Donation after Cardiac Death (DCD) kidneys have increased substantially in recent years [25, 26]. However, the hypothermic perfusion preservation induced injury via cold ischemia/reperfusion is still evitable and may cause acute renal failure (ARF) eventually. The protease inhibitor ulinastatin has been proved to protect many organs, such as heart and kidney from ischemia/reperfusion-induced injury. However, the protective effects of ulinastatin on cold ischemia/reperfusion induced preservation injury and its underlying mechanism have not been reported so far.

In the present study, our results showed that ulinastatin was observed with the protective effects on renal cold ischemia/reperfusion induced injury, as proved by the decreased serum BUN and Cr level. Further results, such as mRNA and Western blotting analysis, histologic staining, indicated that protective effects of ulinastatin on renal cold I/R injury were also closely related to its regulating capability of the microenvironment, such as decreased acute inflammatory response, oxidative stress damage and apoptosis, as well as attenuation of vasculature levels decrease. The present study indicated that ulinastatin could reduce preservation injury induced by cold I/R during renal transplantation surgery.

Previously, the ulinastatin was proved to attenuate I/R-induced injury in many major organs, including lung, heart and liver. Chen's paper also proved that ulinastatin can reduce the renal dysfunction and injury associated with ischemia–reperfusion of the kidney under normal condition. The protective effect of ulinastatin was indicated to be associated with its regulation effects on anti-apoptosis and membrane stability [20]. In this paper, the decreased serum BUN and Cr level data confirms that ulinastatin can also protect the renal from cold ischemia/reperfusion-induced injury, which make it a potential valuable clinical candidate for application during kidney organ preservation.

Acute inflammatory response is the early response of the renal ischemia–reperfusion (I/R) injury [4, 27]. Studies have shown that the ischemia–reperfusion (I/R) injury promotes the formation of some acute inflammatory cytokines that may contribute to the renal dysfunction, necrosis and apoptosis during I/R injury. The mediators, including the two most important cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), appear to specific target endothelium and neutrophils to induce obstruction of capillary beds, which might eventually lead to no-reflow phenomenon of renal tissue during reperfusion [28, 29]. The activation of both TNF-α and IL-6 was proved to be able to regulate the functions of receptors on the cell membrane surface, and to alter the cytosolic protein synthesis and kinases activation status [30, 31]. In the present study, the cold I/R-induced renal injury were observed with significantly increase of the TNF-α and IL-6 mRNA expression levels, which were attenuated by ulinastatin treatment.

Oxidative stress refers to the status of reactive oxygen species (ROS) overproduction due to disruption of the intracellular reduction–oxidation (redox) balance [32]. Large numbers of studies have proved that oxidative stress is involved in pathogenesis of I/R injury [33]. Normally the overproduced ROS would impair membrane lipids, cellular structures, proteins, and DNA of cells, which eventually lead to cellular apoptosis and dysfunction. Malondialdehyde (MDA) is a biomarker of lipid peroxidation and thus could be used as an indicator of oxidative stress levels. Superoxide dismutase (SOD) also plays a vital role in converting ROS to innocuous substance [34]. Therefore, we used both MDA and SOD to determine the antioxidant properties of ulinastatin on renal I/R injury. As expected, the cold I/R injury significantly increased the level of MDA and decreased the SOD activity, while the damage was attenuated via ulinastatin administration in cold I/R + ulinastatin group. These results suggested that ulinastatin could also suppress the oxidative stress damage during I/R injury process.

Both necrosis and apoptosis were proved to be the major contributors to I/R-induced cell death [35–37]. Apoptosis refers to the form of programmed cell death, which involved in the activation of caspase family such as caspase 3 and caspase 7. The renal necrosis and apoptosis was therefore determined by TUNEL assay, and the protein contents of apoptosis-related proteins such as caspases and Bcl family were also evaluated [38, 39]. The results suggested that cold I/R injury induced unfavorable microenvironment resulted in the high level of the TUNEL-positive renal cells number, which was closely related with high levels of the cleaved caspase 3 and 7, Bcl-xl as well as decreased Bcl-2. But the I/R injury-induced damage could partly be restored by ulinastatin treatment. CD31 and α-SMA expression analysis results showed that the ulinastatin could also attenuate the injury of renal vasculature induced by cold I/R. In short, our results revealed that could attenuate the cold I/R-induced renal injury via ameliorating its local unfavorable microenvironment.

In conclusion, the protective effects of ulinastatin on cold renal ischemia–reperfusion injury in rats were reported. Ulinastatin was observed to mitigate the preservation injury induced by hypothermic perfusion. In addition, the attenuated kidney cold I/R injury by ulinastatin was also accompanied with its regulating capability of the microenvironment, such as decreased acute inflammatory response, oxidative stress damage and apoptosis, as well as attenuation of vasculature levels decrease. The present study suggested that ulinastatin may be clinically useful in reducing preservation injury induced by cold I/R during renal transplantation surgery.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests in the present study.

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