Effects of ulinastatin on renal ischemia-reperfusion injury in rats

Cong-cong CHEN[1], Zi-ming LIU, Hui-hua WANG, Wei HE, Yi WANG, Wei-dong WU

Department of Anesthesiology, Second Affiliated Hospital, Medical College of Zhejiang University, Hangzhou 310009,China

KEY WORDS reperfusion injury; urinastatin; immunohistochemistry; ultrastructure

ABSTRACT

AIM: To investigate the effect and possible mechanism of ulinastatin on renal ischemia-reperfusion injury in rats. **METHODS:** Male Sprague-Dawley rats were subjected to 45-min bilateral renal ischemia, treated with intravenously 12 500 U ulinastatin at 30 min prior to ischemia and at the beginning of reperfusion, compared with a nontreated group without ulinastatin and a sham-operation group without bilateral renal ischemia. After 0 h, 2 h, 6 h, 12 h, and 24 h of reperfusion, serum creatinine and blood urea nitrogen were measured for the assessment of renal function, renal sections were used for histologic grading of renal injury, for immunohistochemical localization of Bcl-2 and heat shock protein 70. Renal ultrastructure was observed through a transmission electron microscope. **RESULTS:** Ulinastatin significantly reduced the increase in blood urea nitrogen and creatinine produced by renal ischemia-reperfusion, suggesting an improvement in renal function. Ulinastatin reduced the histologic evidence of renal damage associated with ischemia-reperfusion and accompanied with an up-regulation in the expression of Bcl-2 protein, but it had no significent effect on the expression of HSP 70. Ulinastatin also significantly reduced kidney ultrastructure damage caused by renal ischemia-reperfusion. **CONCLUSION:** The protease inhibitor, ulinastatin, reduced the renal dysfunction and injury associated with ischemia-reperfusion of the kidney. The protective effect of ulinastatin might be associated with the up-regulation of Bcl-2 expression and the effect on membrane fragility.

INTRODUCTION

It was necessary to block renal circulation temporarily in some complicated renal operations. Ischemic injury of renal tubular cells could cause acute renal failure (ARF). Ulinastatin, a potent protease inhibitor, had been proven to have protective effect on many organs[1]. Recent studies showed that ulinastatin had therapeutic effects on experimental crescentic glomerulo-nephritis in rats[2]. It was also reported that ulinastatin could increase urine volume in rats[1]. However, the effects of ulinastatin on renal ischemia-reperfusion injury were rarely reported. The present study was designed to investigate the effects of ulinastatin on serum creatinine, blood urea nitrogen, histologic grading, expression of Bcl-2 and heat shock protein (HSP) 70, and kidney ultrastructure in renal ischemia-reperfusion injury, and try to explore the possible mechanism of ulinastatin on anti-ischemic injury.

MATERIALS AND METHODS

Drugs and reagents Ulinastatin, extracted by Guangdong Techpool Biochemical Company. HSP 70

[1] Correspondence to Cong-cong CHEN. Phn 86-571-8778-3648. Fax 86-571-8778-3638. E-mail ccc@hz.cn
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and Bcl-2 monoclonal antibodies were purchased from Fuzhou Maixin Biological Technology Company.

Grouping Seventy-five Sprague-Dawley rats (male, 230±20 g, Grade II, Certificate No 220010014, provided by Experimental Animal Center of Medical College of Zhejiang University) were randomly divided into 3 groups: sham-operation, ischemia-reperfusion (I-R), and I-R pre-treated with ulinastatin. In the ulinastatin group, 12 500 U ulinastatin was intravenously given at 30 min prior to ischemia and the beginning of reperfusion. Each group included 5 parts according to reperfusion duration: 0 h, 2 h, 6 h, 12 h, and 24 h. All animals were housed at a temperature of 18-20 ºC, humidity of 65 %-69 %, and were submitted to a 12 h light-dark cycle. Rats had unrestricted access to tap water and standard rat chow.

Model I-R group: anesthesia was induced with ketamine 50 mg/kg intraperitoneally. The animals were placed on a heating mat, to keep the body temperature at 36±1ºC measured by a rectal probe attached to a Datex monitor. The vein in the tail was cannulated for fluid and drug administration. A midline laparotomy was performed, both kidneys were located, and the renal pedicles, containing the artery, vein, and nerve supplying each kidney, were carefully isolated. Rats were allowed to stabilize for 30 min before they were subjected to bilateral renal pedicles[3]. Once reperfusion commenced the artery clips were removed. Occlusion was verified visually by change in the color of the kidneys to a paler shade and reperfusion by a blush. Sham-operation group: rats underwent identical surgical procedures as I-R group without bilateral renal clamping.

Experimental protocols Left nephrectomy were performed at 0, 2, 6, 12, and 24 h after reperfusion and blood samples were collected from the heart, serum concentration of urea and creatinine were measured. 2/3 of each kidney samples were fixed in 10 % formalin, followed by routine praffin embedding. Tissue sections were cut (4 μm) and stained with hematoxylin and eosin (HE) for histologic grading of renal injury, scored with semi-quantitative scale evaluating changes by paller's standard[4] . Briefly, paraffin tissue sections were placed on glass slides. Endogenous peroxidase activity was blocked with 1:50 normal horse serum to suppress nonspecific background staining. Then, the primary antibody, rabbit anti-mouse Bcl-2 polyclonal antibody, or rabbit anti-mouse HSP 70 polyclonal antibody was applied respectively. For negative control sections, PBS was used in place of primary antibody. Positive control sections were supplied by Fuzhou Maixin Biological Technology Company. Staining was performed by avidin-biotin complex (ABC) method. The sections were examined using a Olympus-HB2 standard light microscope performed at high power (×400). By counting the number of positive stained kidney cells in 5 high-power fields per case, we determined positive rate of Bcl-2 and HSP 70[5]. The standards for Bcl-2 and HSP 70 quantification were 0 %, 0 point; <25 %, 1 point; 25 %-50 %, 2 points; 50 %-75 %, 3 points; >75 %, 4 points. One third of each kidney samples were fixed by 2.5 % phosphate buffered glutaraldehyde, prepared for observation at transmission electron microscope of Philips TECNAL-10.

Statistical analysis Data were expressed as mean±SD. The significant differences between groups were tested by analysis of variance and *H*-test using SPSS 11.0 statistic software.

RESULTS

Effect of ulinastatin on serum BUN and Cr level BUN and Cr level after 12-h and 24-h reperfusion in ulinastatin group was markedly lower than that in I-R group ($P<0.05$) (Tab 1).

Effect of ulinastatin on histologic evidence of renal damage Morphologic abnormalities including cytoplasmic vacuolization, cell necrosis of the proximal convoluted tubule, and tubular lumen obstruction and impairments were found commonly in I-R group. There were cloudy swelling, granular degeneration, cell necrosis, and lumen obstruction found uncommonly in ulinastatin group. The mean histologic score by standard of paller in ulinastatin group was markedly lower than that in I-R group at the reperfusion point of 0, 6, and 24 h ($P<0.05$) (Tab 2, Fig 1).

Effects of ulinastatin on Bcl-2 and HSP 70 expression The Bcl-2 expression increased in ulinastatin group. As compared with the I-R group, Bcl-2 expression in ulinastatin-treated group increased significantly at 6 h after reperfusion (2.00±1.21 *vs* 0.80±0.64) and at 24 h after reperfusion (2.20±0.84 *vs* 1.20±0.84) ($P<0.05$). But ulinastatin seemed to have no effect on HSP70 expression (Tab 3, Fig 2, 3).

Effect of ulinastatin on ultrastructure of kidney Transmission electron microscope showed that in I-R group, the microvilli of the proximal tubule cells were short and scare, part of the membrane even became smooth; injured mitochondria were obviously

Tab 1. The effect of ulinastatin on BUN and Cr content in serum in renal ischemia-reperfusion injury of rats. *n*=5. Mean±SD. [b]*P*<0.05 *vs* sham-operation group. [e]*P*<0.05 *vs* I-R group.

Group	0 h	2 h	6 h	12 h	24 h
BUN/mmol·L^{-1}					
Sham-operation	7.7±1.6	7.6±1.2	4.9±1.5	5.9±0.3	5.4±0.6
I-R	8.6±1.8	14.5±2.0[b]	20.8±0.9[b]	22±5[b]	21±7[b]
Ulinastatin	8.4±1.7	13.9±2.2[b]	19.1±2.4[b]	16±3[be]	14.0±2.6[be]
Cr/μmol·L^{-1}					
Sham-operation	62±18	53±12	57±17	77±10	63±10
I-R	93±16[b]	100±17[b]	151±11[b]	173±35[b]	167±39[b]
Ulinastatin	81±6	103±19[b]	143±9[b]	130±30[be]	116±13[be]

Tab 2. The effect of ulinastatin on Paller index in renal ischemia-reperfusion injury of rats. *n*=5. Mean±SD. [b]*P*<0.05 *vs* sham-operation group. [e]*P*<0.05 *vs* I-R group.

Group	0 h	2 h	6 h	12 h	24 h
Sham-operation	15.0±2.4	50±34	10±4	25±18	50±4
I-R	85±14[b]	90±28	130±45[b]	115±49[b]	200±40[b]
Ulinastatin	15±4[e]	45±21	85±28[be]	110±14[b]	105±21[be]

Tab 3. The effect of ulinastatin on HSP 70 and Bcl-2 index in renal ischemia-reperfusion injury of rats. *n*=5. Mean±SD. [b]*P*<0.05 *vs* sham-operation group. [e]*P*<0.05 *vs* I-R group.

Group	0 h	2 h	6 h	12 h	24 h
HSP 70					
Sham-operation	0.00±0.00	0.00±0.00	0.60±0.49	0.00±0.00	0.60±0.35
I-R	0.00±0.00	0.00±0.00	0.20±0.15	0.60±0.25[b]	1.80±0.45[b]
Ulinastatin	0.00±0.00	0.00±0.00	0.60±0.49	1.00±0.61[b]	2.20±0.85[b]
Bcl-2					
Sham-operation	0.20±0.15	1.20±0.84	0.80±0.30	0.40±0.25	0.60±0.34
I-R	0.40±0.35	1.20±0.45	0.80±0.64	1.20±0.84	1.20±0.84
Ulinastatin	0.80±0.45	1.80±0.84	2.00±1.20[be]	1.80±0.84[b]	2.20±0.45[be]

observed, some of the mitochondria became vacular-like; rough endoplasmic reticulums were reduced in number, the figure was enlarged, and some of ribosome disappeared; foamy changes in the structure of nuclear and cytoplasm were also observed. In ulinastatin group, the microvilli were slightly swelled or kept intact; the cristae of the mitochondria were slightly disorgnized but the structure was still intact; rough endoplasmic reticulums were slightly swelled, ribsome was all preserved, nuclear had almost normal figure with very clear nucleolus (Fig 4). Ulinastatin produced protective effect on renal ischemia-reperfusion injury.

DISCUSSION

Ulinastatin, a protease inhibitor, was a glycopro-

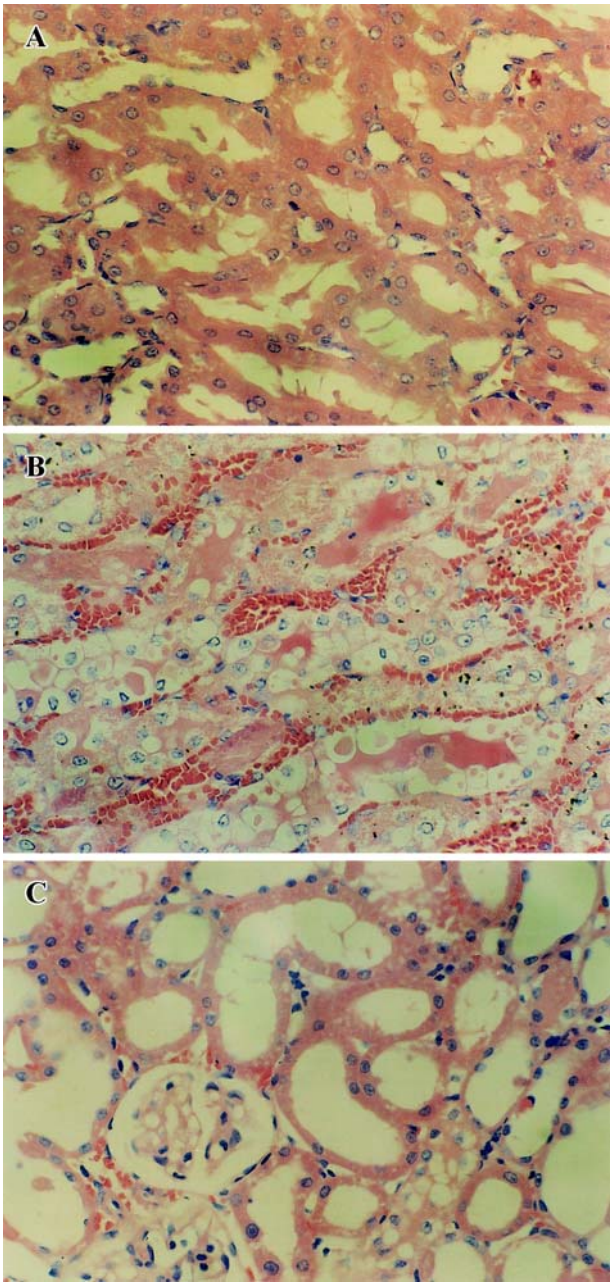

Fig 1. Effects of ulinastatin on mice renal ischemia-reperfusion injury (reperfusion for 24 h). Light microscope, HE stain, ×400. A) sham-operation group. B) ischemia-reperfusion group. C) ulinastatin treated group.

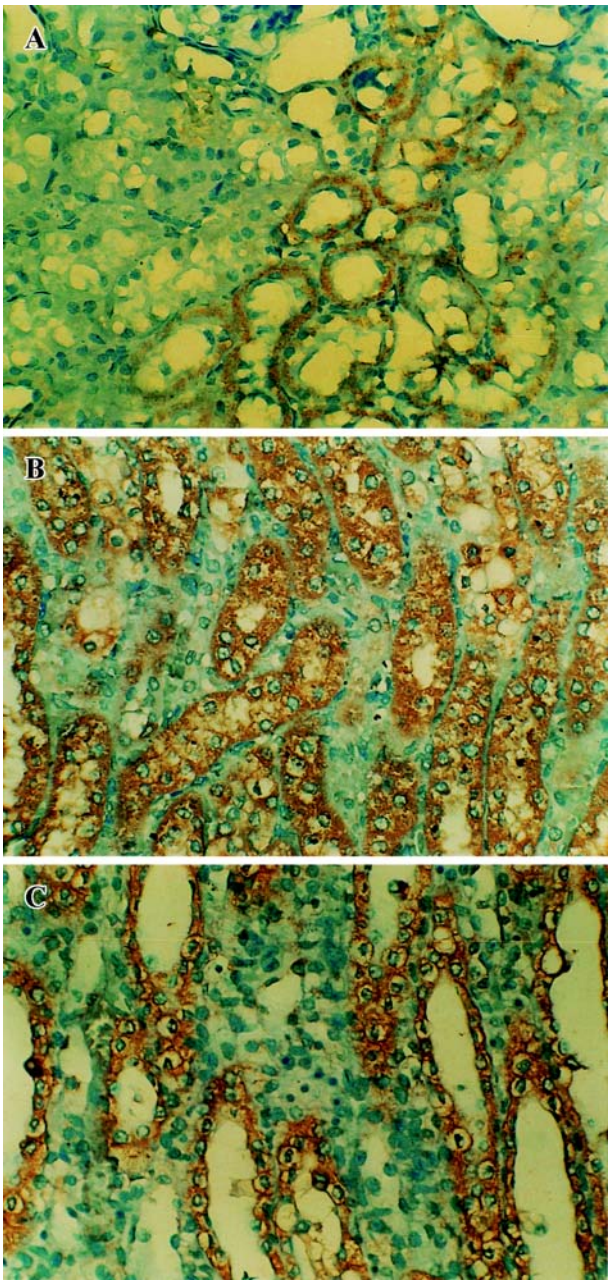

Fig 2. Effects of ulinastatin on HSP 70 protein expression in renal ischemia-reperfusion injury in rats (reperfusion for 24 h). Light microscope, ×400. A) sham-operation group. B) ischemia-reperfusion group. C) ulinastatin treated group. Nuclear or cytoplasma brown staining indicates positive expression.

tein derived from human urine with a molecular weight of 67 kDa[1]. UTI was reported to have a protective effect against hepatic I/R injury *in vivo*, through its mechanisms of membrane stabilization, inhibition of such lysosomal enzymes as elastase and catepsin G[6], and radical scavenging action[7]. However, the effect of ulinastatin on renal ischemia-reperfusion injury has not been reported.

Elevation in free radicals and lipid peroxidation taking place after reperfusion played a key role in the injury associated with ischemia-reperfusion. Superoxide anion was generated during the period of reperfusion after ischemia, and free radical including superoxide and hydroxyl radicals was involved in the damage induced by ischemia-reperfusion injury[8]. Ischemia-reperfusion injured renal tissue was particularly sensitive to oxygen

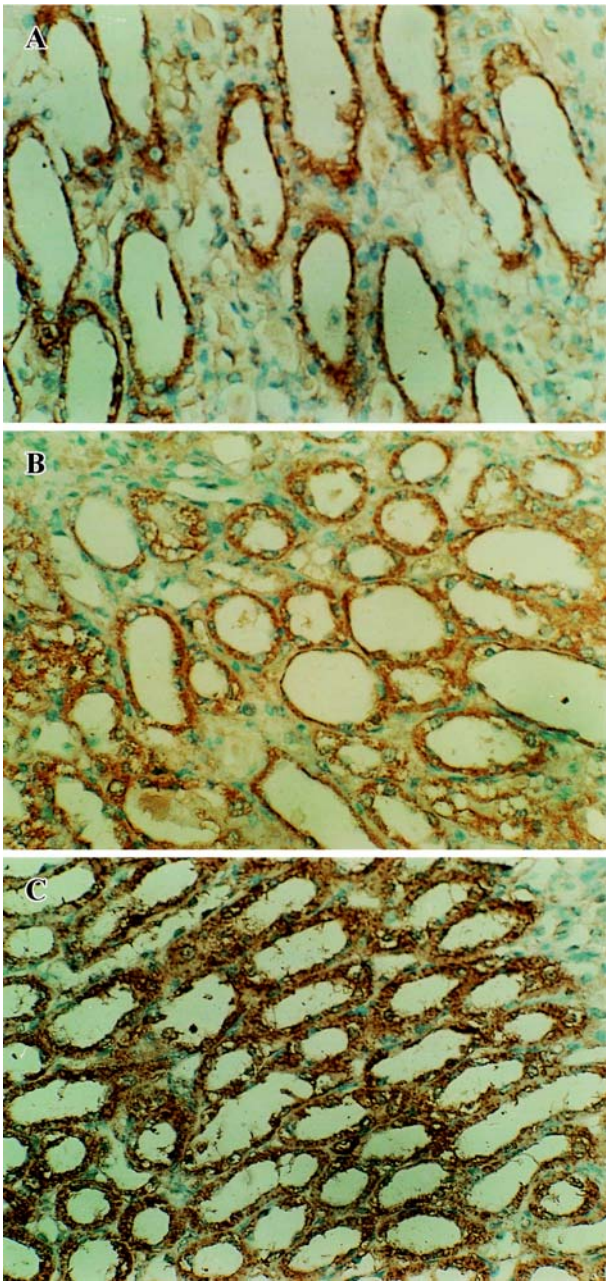

Fig 3. Effects of ulinastatin on Bcl-2 protein expression in renal ischemia-reperfusion injury in rats (reperfusion for 24 h). Light microscope,×400. A) sham-operation group. B) ischemia-reperfusion group. C) ulinastatin-treated group. Cytoplasma brown staining indicates positive expression.

radical-mediated injury due to its low concentrations of O_2 radical scavenging enzymes, and with high levels of polyunsaturated fatty acids that constitute the renal cell membrane and mitochondrial membrane. Mitochondria injury caused the dysfunction in energy metabolism thereby leading to a vicious cycle[9]. Intravital microcirculation study clearly demonstrated that ischemia-reperfusion promoted the adherence of leukocytes to the vascular endothelium. Moreover, elastase, a protease released from the activated neutrophils, was reported to play an important role in modulating the leukocyte-endothelial cell interactions induced by ischemia-reperfusion[6].

Bcl-2 is a kind of inhibitor of the cell apoptosis.It can prevent the apoptosis induced by free radicals and lipid peroxidation. Bcl-2 has the antioxidative characteristics in cells through participating the reduction action and inhibiting the formation of active oxygen. Bcl-2 also preserved mitochondrial oxidative function[10].

In the present study, the protective effect of uliastatin on renal ischemia-reperfusion injury were evaluated by the changes of serum BUN and Cr level, renal histology, expression of Bcl-2 and HSP 70 protein, and ultrastructure of proximal tuble cells. The results showed that serum BUN and Cr level hoiked after 2 h of reperfusion and reached the zenith at 12 h after reperfusion. The mean histologic score by standard of paller increased just at the beginning of reperfusion and reached the zenith at 24 h, after reperfusion. There was no significant difference of Bcl-2 protein expression and HSP 70 protein expression between preischemia and post-reperfusion in renal ischemia-reperfusion model in rats. This was incedenced with previous study[11]. In I-R group, the ultrastructure of proximal tubule cells changed.

After treatment with ulinastatin, serum BUN and Cr levels were lower, mean histologic score by standard of paller was lower, expression of Bcl-2 protein enhanced, but there was no significanly change about expression of HSP 70 protein as compared with I-R group. Ulinastatin could also attenuate injury of mitochondria and rough endoplasmic reticulus. The injury of mitochondria, which were the major site subjected to great injury by ischemia-reperfusion, was significantly improved.

To our knowledge, we demonstrated that administration of ulinastatin prior to ischemia and at the beginning of reperfusion reduced the renal dysfunction and injury caused by ischemia-reperfusion of the rat kidney.

Ulinastatin was found to be effective in reducing renal ischemia-reperfusion injury *in vivo*, which was accompanied with an increased production of Bcl-2 protein. Moreover, the mitochondria is protected by ulinastatin.

Ulinastatin surpressed the release of neutrophil elastase and inhibited the extravasation of neutrophils

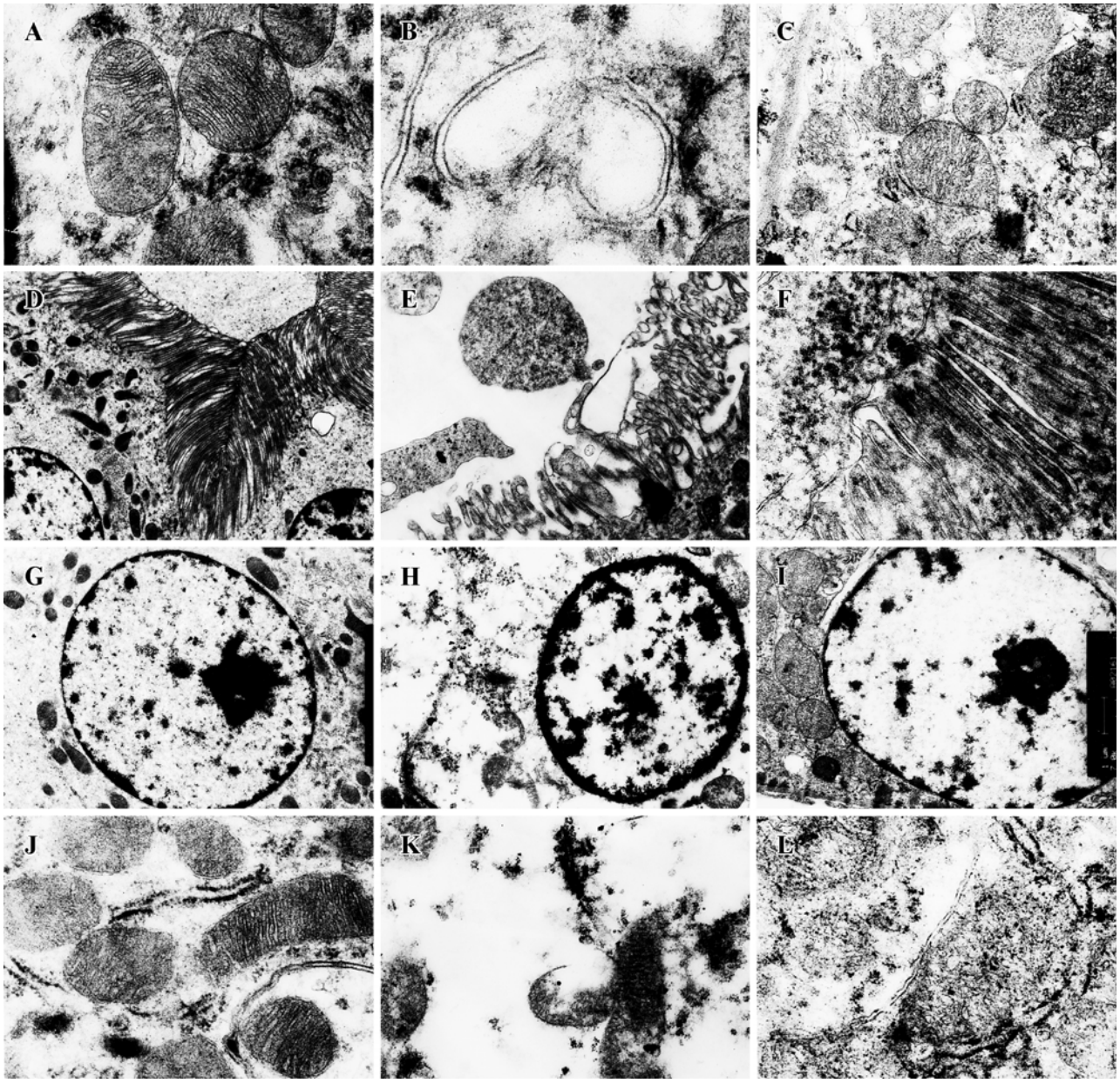

Fig 4. Structure of renal proximal cells after reperfusion for 24 h was observed under teansmission electron microscope. A) structure of mitochondria was normal in sham-operation group (×34 000). B) structure of mitochondria, became vascular-like in I-R group (×44000). C) the cristae of mitochondria was slightly disorganized or unchanged in ulinastatin-treated group(×17000). D) long microvilli were observed in sham-operation group (×3700). E) membrane of cells became smooth in I-R group (×7000). F) a few of microvilli were slightly swelled in ulinastatin-treated group (×17 000). G) structure of nuclear was normal in sham-operation group (×7000). H) there were foamy changes in the structure of nuclear in I-R group (×7000). I) structure of nuclear almost normal in ulinastatin treated group (×7000). J) many rough endoplasmic reticulums (RER) with normal figure and ribosome on them were observed in sham-operation group (×34 000). K) RER were reduced, with enlarged figure, some ribosome disappeared in I-R group (×20 000). L) RER were slightly swelled but not reduced and ribosome were all preserved (×27 000).

through the vascular wall. Ulinastatin also had an inhibitory action on lysosome enzymes such as trypsin, elastase, and lipase, and had a stabilizing effect on lysosomal membranes[6]. It also exhibited a corticosteroid-like effect, seemed not only to protect renal cells in the ischemic kidney by stabilizing cell membranes and intracellular granules, but also to improve renal microcirculation by inhibiting actions of granulocyte elastase and cytokines[12]. Moreover, ulinastatin had a suppressive effect against neutrophil superoxide production[13].

These findings might thus explain our results.

In conclusion, ulinastatin provided remarkable protection against renal ischemia-reperfusion injury in rats. Ulinastatin attenuated kidney failure accompanied with an increase expression of Bcl-2 and without significant change of the HSP 70 expression, which suggested that the up- regulation of Bcl-2 protein expression might be one of the mechanisms of ulinastatin's protective effect on renal ischemia-reperfusion injury, thus plays its role of antioxidation.

Since ulinastatin has already been safely used in clinical trials for other disease states, it may be clinically useful in human renal ischemia-reperfusion injury. It has not been determined, however, whether ulinastatin is similarly effective for nephropyelolithotomy and renal transplantation in human.

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