

## Heparin Attenuated Neutrophil Infiltration but Did Not Affect Renal Injury Induced by Ischemia Reperfusion

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*Although heparin is better known as an anticoagulant, it also has several anti-inflammatory effects. Heparin is known to inhibit neutrophil adhesion, chemotaxis and oxygen free radical production. In addition, heparin is also known to act as an oxygen radical scavenger. Our hypothesis was that heparin would attenuate renal ischemia reperfusion injury. In this study, we investigated whether heparin had a protective effect on renal ischemia reperfusion injury. Sheep (n=12) were prepared for the chronic study with venous, arterial and urinary catheters inserted. In addition, pneumatic occluders and ultrasonic flow probes were placed on renal arteries. After a 5-day recovery period, the sheep were randomized to either a heparin treatment group (400 IU/kg IV bolus 10 minutes before renal artery occlusion, followed by a continuous effusion 25,000 IU in 250 ml of 0.9% NaCl at 10 ml/hr, n=6) or a control group (n=6), which received an equivalent volume of 0.9% NaCl. All the sheep then underwent 90 minutes of bilateral renal ischemia followed by 24 hours of reperfusion. Blood urea nitrogen (BUN), serum creatinine (Scr), and creatinine clearance (CrCl) were determined at various intervals during both the ischemic and reperfusion periods. Kidney tissue samples were obtained at autopsy for histologic examination. As a result, there were significant differences in the degree of inflammation ( $1.50 \pm 1.24$  Vs  $0.50 \pm 0.79$ ,  $P < 0.05$ ) between the control and heparin treatment groups, but not in the degree of injury ( $2.83 \pm 0.44$  Vs  $2.33 \pm 0.28$ ). In this study, heparin significantly attenuated polymorphonuclear leukocytes (PMNs) infiltration within the interstitium, but it did not affect the degree of renal damage as measured by urinary chemistries or renal tubular damage as assessed by histopathologic evaluation.*

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Renal ischemia reperfusion injury is common after aortic surgery in which the suprarenal aorta is cross-clamped and in renal arterial revascularization procedures. Heparin is often administered prior to vascular occlusion in order to prevent thrombosis. Although better known for its anticoagulant effect, heparin has several important antiinflammatory effects, which may be beneficial in the setting of renal ischemia reperfusion injury (Zigmond and Hirsch, 1973). Heparin exerts many of its antiinflammatory effects by acting on PMNs. Heparin has been reported to inhibit PMNs adhesion to the vascular endothelium (Silvestero *et al.* 1994), chemotaxis of PMNs from the vasculature to the interstitium (Matzner *et al.* 1984), and PMNs mediated production of oxygen free radicals. In addition to these effects on PMNs, heparin limits the toxicity of generated oxygen free radicals by releasing extracellular superoxide dismutase (Karlson and Marklund, 1987) and by the possible binding to the ionic site of transitional metals necessary for generation of the OH<sup>-</sup> radical.

The hypothesis of the present study was that these antiinflammatory effects of heparin would be beneficial in the setting of renal ischemia reperfusion injury.

## MATERIALS AND METHODS

The procedures and experimental design described below were approved by the University of Texas Medical Branch-Galveston, Animal Care and Use Committee. During the course of this experiment, we followed the guidelines for the care and use of experimental animals as established by the National Institutes of Health.

Female sheep (n=12) of the Merino breed, weighing 26~44 kg, free of parasites and other infectious agents, were prepared for this study five days before experimentation. After overnight fasting, with free access to water, anesthesia was induced with 2~2.5% halothane via mask. Following induction, the animals were intubated endotracheally and placed on mechanical ventilation. Under 2% halothane anesthesia and sterile conditions, a Swan-Ganz thermal-dilution catheter (model 93A-131-7F, Edward Lab-

oratories, Palo Alto, CA, USA) was inserted into the pulmonary artery via the right common jugular vein. The right groin was surgically prepared and draped in the usual sterile manner, and an incision was made over the right femoral vessels. The femoral artery and vein were identified, isolated and one silastic catheter (Becton/dickinson, Sandy, Utah, USA) was placed in each vessel and advanced into the abdominal aorta and caudal vena cava, respectively. The animal was then positioned and the right flank was prepared and draped in the usual sterile manner. Through retroperitoneal approach, the right renal artery was identified and isolated. A pneumatic occluder (IVM, Healdsburg, CA, USA) was placed around this renal artery near its origin from the aorta. The more distal portion of this artery was also isolated and dissected free from surrounding tissues. A 4 mm transit time ultrasonic flow probe (Transonic System, Ithaca, NY, USA) was placed around this distal portion of the artery and sutured *in situ* to prevent rotation. The flank incision was closed in layers using appropriate sutures. Each animal was then repositioned and the above described procedure was applied to another renal artery. The animals were allowed a five-day period to recover from the anesthetic, during which they were monitored for adequate food, water intake, adequate urine output, temperature and postoperative pain. A urinary catheter was placed under ketamine anesthesia (1.5 mg/kg, IV; Parker Davis, Morris Plains, NJ, USA) 24 hours prior to the start of the experiment.

### Experimental design

Twelve sheep underwent ischemia reperfusion injury in the kidney and were randomized into two groups of six. The heparin group (n=6) received 400 units per kilogram bolus (approximately 20 ml) of porcine heparin (Upjohn, Kalamazoo, MI, USA) 10 minutes before renal occlusion and a continuous heparin infusion (25,000 units per 250 ml of normal saline at 10 ml/hour). The control group (n=6) received a saline solution vehicle.

Standard hemodynamic measurements, urine output, creatinine clearance and blood urea nitrogen concentration were determined at baseline during the ischemic period, and at 0.5, 8, 16 and 24 hours after reperfusion. Renal ischemia induced by inflation of

**Table 1. Definition of terms for description of histologic change in ischemic injury of kidney**

Degree	Ischemic injury	Acute inflammation
0	No detectable injury	No detectable inflammation
+	Foci of tubular necrosis	Minimal, focal infiltration
++	Extensive tubular necrosis	Multiple foci of PMNs
+++	Extensive tubular necrosis and focal necrosis of other tissue	Patch, moderate PMNs infiltration
++++	Large zones of necrosis of all structures	Dense extensive infiltration of PMNs

bilateral renal artery occlusion was obtained by a zero-flow reading with ultrasonic flow probe. After completion of protocol, the animals were anesthetized with ketamine (1.5 mg/kg, IV) and sacrificed with 60 ml of saturated potassium chloride solution. Once death was confirmed by flat pressure trace for 10 minutes, autopsy was performed and samples were collected for renal histology.

#### Standard hemodynamics

Mean arterial, central venous, pulmonary artery and pulmonary capillary wedge pressures were measured using transducers (P231; Statham Gould, Oxnard, CA, USA) with a continuous heparinized flushing mechanism. The transducers were connected to an OM9 physiologic recorder (Electronics for Medicine-Honeywell OM9, Peasantsville, NY, USA) for graphic display of electronically-calculated mean pressures. Cardiac output was determined by thermodilution technique, using a cardiac output computer (Model 9520, American Edwards Laboratories, Irvine, CA, USA). Ten ml of 5% dextrose solution at 0°C was used as an indicator.

#### Renal tissue conjugated dienes assay

We measured the conjugated level of the renal tissue (medulla, cortex) as an indicator of reperfusion injury. The procedure for measuring kidney tissue conjugated dienes has been previously described by Till *et al.* (1985).

#### Renal histology

Specimens of both right and left kidneys were

harvested for light microscopy. These samples were fixed in 10% buffered formalin, embedded in paraplast, sectioned at 4 mm intervals, and stained with hematoxylin and eosin as well as Leder's stain to extenuate the presence of neutrophils. The extent of ischemic injury and degree of inflammation was graded on a semiquantitative scale (Table 1) of 0 to 4+ that reflected the increasing degrees of ischemic damage. All slides were interpreted by a pathologist blinded to the duration of ischemia.

#### Statistical analysis

Hemodynamic and blood sample data of the heparin treatment group and control group were compared with each other and the baseline values which served as control data. The control and experimental kidneys for each set of experiments were contrasted. To assess renal injury, the sections were coded and the extent of ischemic injury and degree of neutrophil infiltration were graded on a semi-quantitative scale of 0 to 4+ that reflected the increasing degree of ischemic damage and the degree of neutrophil infiltration as described in the Results. The repeated measures ANOVA was used to determine the difference at each time period compared to baseline values. Comparison between the groups was made by paired Student's t-test with Bonferroni correction. Statistical significance was set at  $p < 0.05$ .

## RESULTS

All animals underwent surgical preparation without postoperative complication. In particular there

**Table 2. Hemodynamic changes during ischemia reperfusion injury in kidney (mean  $\pm$  SE)**

Variable	Group	Reperfusion					
		Base	Ischemia	0.5 hours	8 hours	16 hours	24 hours
CI	C	6.89 $\pm$ 0.61	5.59 $\pm$ 0.71	6.07 $\pm$ 0.73	6.08 $\pm$ 0.45	5.85 $\pm$ 0.46	6.09 $\pm$ 0.33
	H	7.00 $\pm$ 0.34	5.84 $\pm$ 0.54	5.81 $\pm$ 0.46	6.47 $\pm$ 0.34	5.74 $\pm$ 0.41	5.65 $\pm$ 0.26
MAP	C	93 $\pm$ 6.1	116 $\pm$ 9.1	120 $\pm$ 8.1*	111 $\pm$ 7.8	106 $\pm$ 5.1	100 $\pm$ 5.9
	H	101 $\pm$ 8.1	132 $\pm$ 10.0*	121 $\pm$ 9.0	106 $\pm$ 6.4	105 $\pm$ 9.4	104 $\pm$ 8.5
PAP	C	19.5 $\pm$ 1.7	21.5 $\pm$ 1.5	21.0 $\pm$ 1.5	22.8 $\pm$ 1.9	22.6 $\pm$ 1.3	22.1 $\pm$ 1.8
	H	17.8 $\pm$ 1.3	20.0 $\pm$ 1.4	19.3 $\pm$ 1.4	19.5 $\pm$ 2.8	18.3 $\pm$ 1.4	19.3 $\pm$ 1.5
PCWP	C	7.0 $\pm$ 0.8	8.5 $\pm$ 0.6	9.5 $\pm$ 0.9	10.0 $\pm$ 1.5	9.6 $\pm$ 0.9	10.5 $\pm$ 1.1
	H	7.6 $\pm$ 0.8	10.0 $\pm$ 1.1	7.1 $\pm$ 0.9	8.0 $\pm$ 1.3	5.8 $\pm$ 1.2	6.6 $\pm$ 1.6
SVRI	C	1053 $\pm$ 125	1789 $\pm$ 406	1656 $\pm$ 288*	1391 $\pm$ 130	1368 $\pm$ 123	1225 $\pm$ 94
	H	1112 $\pm$ 116	1781 $\pm$ 195*	1634 $\pm$ 178*	1284 $\pm$ 106	1448 $\pm$ 130	1456 $\pm$ 118

C(n=6); control group, H(n=6); heparin treatment group, CI; cardiac index, MAP; mean arterial pressure(mmHg), PAP; mean pulmonary arterial pressure(mmHg), PCWP; pulmonary capillary wedge pressure(mmHg), SVRI; systemic vascular resistance index(dyne sec cm<sup>-5</sup>/m<sup>2</sup>), \*: compare to base p<0.05

was no evidence of renal dysfunction during the recovery period. All animals survived for the duration of the experiment.

### Hemodynamic alteration

Occlusion of renal arteries resulted in a significant increase in mean arterial pressure and systemic vascular resistance in both groups. There was no significant difference between the control and heparin treatment groups in hemodynamic change during ischemia reperfusion. The increase of mean arterial pressure and systemic vascular resistance may have resulted from activation of the renin angiotensin system after the renal arteries were clamped. Mean pulmonary artery pressure and pulmonary capillary wedge pressure were not significantly increased after renal artery occlusion in both groups (Table 2).

### Renal function

Animals in both the control and heparin treatment groups exhibited the typical features of acute renal failure. There was a progressive increase in serum creatinine, serum BUN and a decline in creatinine clearance in both groups. There was a wide variation

in urine output between each animal in both groups. Although the heparin treatment group showed higher urine output feature until 16 hours after reperfusion, there was no significant difference compared to the control group in urine output at 24 hours. There was no significant difference between the control and heparin treatment groups, except in creatinine clearance at 4 hours after reperfusion (Table 3).

### Renal tissue conjugated dienes

Conjugated diene levels of the renal medulla in both the control and heparin treatment groups were significantly higher than those of the renal cortex (control group: medulla Vs cortex; 3.88  $\pm$  0.15 Vs 3.39  $\pm$  0.09 O.D, and heparin treatment group: medulla Vs cortex; 2.71  $\pm$  0.10 Vs 2.33  $\pm$  0.07, p<0.05). The conjugated diene levels of the medulla and cortex in the heparin treatment group were significantly lower than those in the control group (Fig. 1).

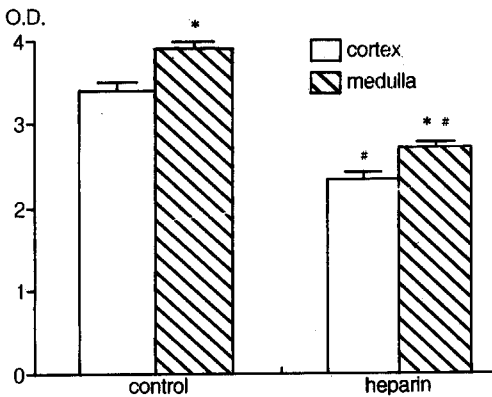
### Pathology

In the control group, the kidneys showed extensive tubular necrosis with caryolysis of most tubular

**Table 3. Renal function during ischemia reperfusion injury (mean  $\pm$  SE)**

Variable	Group	Reperfusion				
		Base	0.5 hours	8 hours	16 hours	24 hours
S-Cr	C	0.90 $\pm$ 0.10	1.98 $\pm$ 0.15*	2.66 $\pm$ 0.19*	3.51 $\pm$ 0.17*	4.05 $\pm$ 0.22*
	H	0.86 $\pm$ 0.04	1.53 $\pm$ 0.07	2.01 $\pm$ 0.13*	2.61 $\pm$ 0.28*	3.10 $\pm$ 0.41*
S-BUN	C	14.8 $\pm$ 2.2	25.1 $\pm$ 3.2	27.1 $\pm$ 6.2	45.5 $\pm$ 5.1	58.1 $\pm$ 5.5
	H	19.1 $\pm$ 2.1	28.3 $\pm$ 3.3	36.1 $\pm$ 4.3	45.3 $\pm$ 5.7	51.1 $\pm$ 6.2
CrCL	C	68.7 $\pm$ 6.8	0.3 $\pm$ 0.2*	0.7 $\pm$ 0.4*	4.0 $\pm$ 1.8*	4.0 $\pm$ 1.7*
	H	67.6 $\pm$ 6.7	15.1 $\pm$ 3.9*, +	14.8 $\pm$ 5.2*	14.0 $\pm$ 5.3*4	13.6 $\pm$ 5.5*
U/O	C	2.5 $\pm$ 0.2	0.3 $\pm$ 0.2*	1.0 $\pm$ 0.5	1.4 $\pm$ 0.7	1.5 $\pm$ 0.7
	H	3.6 $\pm$ 0.4	0.4 $\pm$ 0.1*	4.0 $\pm$ 0.8*	4.0 $\pm$ 0.8*	3.6 $\pm$ 0.7

C(n=6): control group, H(n=6): heparin treatment group, S-Cr(mg%); serum creatinine, S-BUN(mg%); serum blood urea nitrogen, CrCL(ml/min); creatinine clearance. U/O(ml/kg/hr); urine output, \*:  $p < 0.05$  versus base, +:  $p < 0.05$  compare to control group



**Fig. 1.** The optical degree (O.D.) of renal tissue conjugated dienes. O.D. is measured by the degree of absorbance at 233 nm. Values are means  $\pm$  SE (n=12; \* $p < 0.05$  when compared to cortex, \*\* $p < 0.05$  when compared to control).

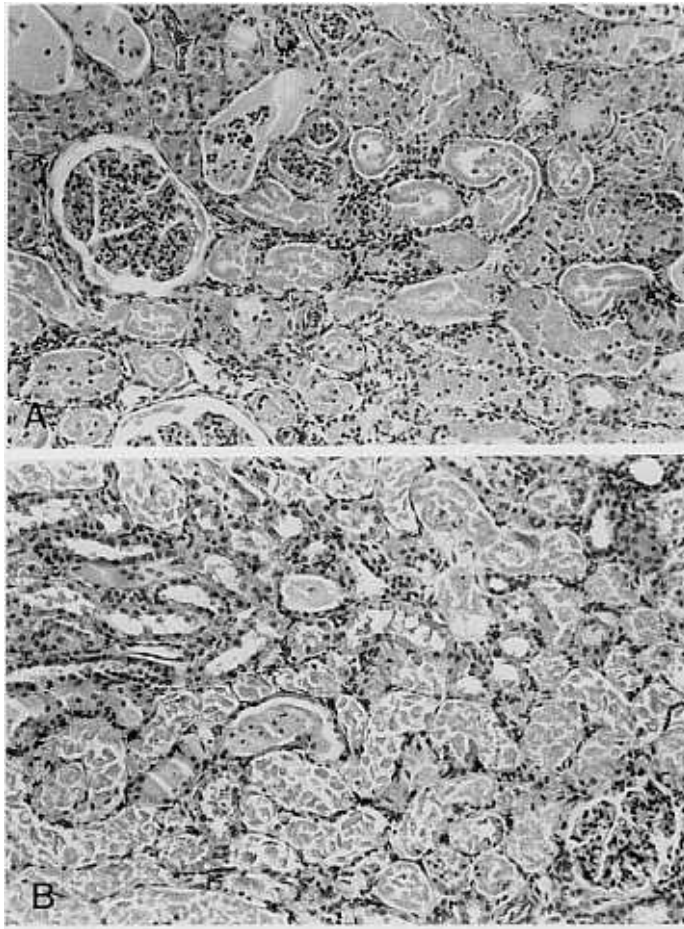
segments in the cortex, with mild-to-moderate dense acute inflammatory infiltrates which were most prominent in the outer cortex and were mostly interstitial, partly tubular.

In the heparin treatment group, animals demonstrated extensive tubular necrosis and little or no acute inflammatory reaction (Fig. 2). There was no significant difference in the degree of tissue damage between the control and heparin treatment groups

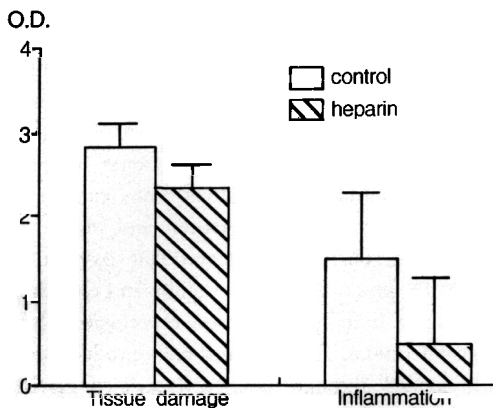
(2.83  $\pm$  0.44 Vs 2.33  $\pm$  0.28). There was a significant difference in the degree of inflammation between the control and heparin treatment groups (1.50  $\pm$  1.24 Vs 0.50  $\pm$  0.79,  $p < 0.05$ )(Fig. 3).

## DISCUSSION

There are a number of mechanisms by which neutrophils can be activated by the kidney during ischemia. It has been reported that neutrophil and oxygen free radicals have important roles in the pathogenesis of ischemia reperfusion injury (Granger, 1988). An activating stimulus could lead to microcirculatory entrapment of granulocytes and result in oxygen radical production (McCord, 1985; Fischbein, 1990). Free radicals are simple molecules with an odd number of electrons. They cause membrane injury by lipid peroxidation and inactivation of enzymes. There is evidence that oxygen free radicals are produced by neutrophils and endothelial cells during both ischemia and reperfusion (Freischlag and Hanna, 1991). Neutrophils produce superoxide anions through their cell surface NADPH oxidase system (Rossi, 1986). The mechanism by which heparin inhibits neutrophil NADPH oxidase to generate superoxide is not known. It has been



**Fig. 2.** H & E stain of renal tissue after 90 minutes ischemia followed by reperfusion. A(control) and B(heparin treatment) both show extensive tubular necrosis, but there are little or no acute inflammatory reactions in B.



**Fig. 3.** Semiquantitative scale of renal tissue injury and inflammation. Values are means  $\pm$  SE ( $n=12$ ;  $*p < 0.05$  when compared to control).

thought that heparin inhibited the neutrophil activation by protein kinase C inhibition (Reisenberg *et al.* 1995).

Heparin is able to induce a prompt increase in plasma EC-SOD activity (Karlson and Marklund, 1987). Evidence has been provided that endothelial cells may be protected in vitro from mediated cytotoxic effects by superoxide dismutase and catalase (Sacks *et al.* 1978). Extracellular superoxide dismutase (EC-SOD) is the major superoxide dismutase isoenzyme in an extracellular fluid like plasma, lymph and synovial fluid. EC-SOD occurs in higher concentration in tissue than in plasma. Therefore, heparin may attenuate tissue injury due to oxygen

free radicals and preserve organ function. However, the wide diversity of EC-SOD in the vascular system of mammals with regard to the total distribution between plasma and endothelium indicates that the pathogenic potential of superoxide radicals in the extra space may vary a great deal between species (Karlson and Marklund, 1988).

Heparin has been known for the inhibition of chemotaxis, activation of neutrophils (Matzner *et al.* 1984) and reduction of the production of oxygen free radicals in neutrophils (Reisenberg *et al.* 1995). Oxygen free radicals are important factors in the activation of neutrophils. Welbourn suggested that ischemia reperfusion injury is initiated by the production of reactive oxygen species which initially appear responsible for the generation of chemotactic activity in neutrophils (Welbourn *et al.* 1991). It has been shown that heparin inhibited both the random migration and directed locomotion of human neutrophils. It resulted from the inhibition of C<sub>5</sub>A, which is the principal chemotactic factor in activated serum (Jaques, 1979).

It has been suggested that large doses of heparin significantly reduce the cellular destruction pathophysiology of myocardial ischemia, burns, thromboembolism and pulmonary infarction in which a role for granulocyte has been confirmed, and the beneficial effects of heparin are dose-related and the effective dose is much greater than that required to produce anticoagulation (Pasini *et al.* 1984).

Commercially available heparin is a combination of compounds with molecular weights ranging from 3,000 to 30,000 daltons. Only one-third of the molecules bind to antithrombin III, which is mainly responsible for the anticoagulant effect. As postulated, we investigated anticoagulant properties independently (Meyer *et al.* 1978). Titrating the heparin dose according to parameters such as clotting time is not necessarily appropriate (Hirsch, 1991). We had an experience of the antiinflammatory effect of heparin in a smoke-inhalation injury of the lung. So we determined dose of the heparin as same as Cox *et al.* (1993).

In this experiment we found a significant decrease of renal tissue conjugated dienes in the heparin treatment group. However, we could not find any conclusive evidence that heparin was able to attenuate the ischemia reperfusion injury in a kidney his-

tologically or functionally. There are several potential explanations for discrepancies between the decrease of tissue conjugated dienes and pathologic and functional improvement of the kidney.

First, it has been shown that the xanthine oxidase inhibitor allopurinol prevents post-ischemic renal injury (Chatterjee and Berne, 1976). We interpreted those studies to suggest that cytoplasmic xanthine oxidase was the major source of oxygen free radicals after renal ischemia. These observations would suggest that neutrophils are not necessary to produce postischemic free radical mediated renal injury because neutrophil NADPH oxidase would not be inhibited by allopurinol. In our study, heparin inhibited neutrophil activation but we could not find definite attenuation of ischemic reperfusion injury. That would suggest that the intravascular site of oxygen free radicals from neutrophils is not critical to postischemic injury.

Second, the kidney is very different from the heart in terms of the sites and courses of postischemic injury, even though oxygen free radical mediated injuries affect both organs. In the heart, neutrophils appear to produce oxygen free radicals from a site in or close to endocardial capillary endothelium. Myocardial xanthine oxidase may or may not be an important source of oxygen free radicals after ischemia reperfusion injury (Schmid-Schönbein and Engler, 1986). In the kidney, an intravascular site of oxygen free radical production and the role of neutrophils are relatively unimportant. Production of oxygen free radicals in the urinary space or along the border membrane of proximal epithelial cells adjacent to the urinary space is of far greater importance. In the kidney, free radicals are generated in or near the urinary space, so free radical scavengers which undergo glomerular filtration and/or tubular secretion (superoxide dismutase, dimethyl thiourea, dimethyl sulfide, glutathione) are protective. Catalase, which is too large to be filtered (Paller *et al.* 1988) was not protective. The blood-flow pattern may have been altered by an ischemic insult compromising tissue delivery of plasma SOD, induced by heparin. If plasma SOD induced by heparin was not delivered in a sufficient dose into the renal tubule during the reperfusion period, one would anticipate that its effects on preventing oxygen free radical damage would be limited.

Third, 90 minutes of ischemia may have exceeded the potential "therapeutic window" for heparin as a neutrophil inhibitor and oxygen free radical scavenger in this experimental model. After a long period of ischemia, the ischemic injury itself is so massive that the contribution of reperfusion is insignificant. Although it has been reported that total occlusion of the renal artery for 60 minutes resulted in a reversible injury in a rat, clamping for more than 90 minutes may produce irreversible disruption of the kidney (Brenner and Lazurus, 1988). In our preliminary study, we could not find neutrophil infiltration in sheep kidneys after 60 minutes of renal artery clamping, but after 90 minutes of renal artery clamping, we found neutrophil infiltration in sheep kidneys. So we determined the renal artery clamping time to be 90 minutes.

Fourth, this result suggested that neutrophils may not have such an important role in renal ischemia reperfusion injury. It has already been shown that neutrophils by  $O_2$  metabolite-dependent mechanism contribute to ischemia reperfusion injury in isolated perfused kidneys (Linass *et al.* 1988). However, it has also been shown that neutrophils and neutrophil adherence are not critical in producing *in vivo* renal ischemia reperfusion injury (Thornton *et al.* 1989). The role of neutrophils in renal ischemia reperfusion injury is unsettled.

And last, in this experiment we observed the sheep 24 hours after reperfusion. Even though there was no statistical or clinical significance between the control and heparin treatment groups at the 24-hour parameter (BUN, serum creatinine, creatinine clearance, tissue damage degree), that shows renal function tended to be better in the heparin treatment group than in the control group. To observe the drug effect of attenuation of ischemia reperfusion injury in the kidney, a longer period of observation may be necessary.

In conclusion, heparin attenuated neutrophil infiltration but it did not show any significant protective effect in ischemia reperfusion injury in the kidney.

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