

Basic Science: First Prize

Erythropoietin-Induced Optimization of Renal Function After Warm Ischemia

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Abstract

Background and Purpose: Recent preclinical data have indicated that erythropoietin (Epo) can protect organs from ischemic damage. We evaluated the ability of Epo to protect the kidney from the effects of ischemia.

Methods: Thirty dogs underwent a laparoscopic nephrectomy and were allowed to recover for 2 weeks. The dogs were then divided into five groups. Animals in groups 1 and 2 underwent 1.5 hours of abdominal insufflation with placebo (saline) injection (group 1) or Epo injection (group 2) before; groups 3 to 5 underwent 1 hour of laparoscopic renal artery clamping after placebo injection (group 3), Epo injection (group 4), or mannitol injection (group 5). Serum evaluations and 24-hour urine collections were performed weekly. After 28 days, the animals were sacrificed. Statistical analysis was performed with the Kruskal-Wallis test.

Results: After recovery from the initial nephrectomy, all dogs had similar serum hematocrit and creatinine levels. Hematocrit was not significantly affected by Epo administration at any time point. Immediately after the second surgery, dogs that underwent renal artery clamping (groups 3–5) had significantly lower 24-hour urine creatinine levels than those that were not clamped (groups 1–2). After 4 weeks of recovery, the dogs that had received Epo before ischemia (group 4) had recovered significantly more renal function than the dogs that received placebo or mannitol before ischemia (urine creatinine level = Epo 149.1 mg/dL *v* placebo 70.7 mg/dL *v* mannitol 80.7 mg/dL). At sacrifice, microalbuminuria was also significantly less in dogs receiving Epo before ischemia than their mannitol or placebo counterparts.

Conclusion: The current study demonstrates that administering Epo before warm ischemia can improve the recovery of renal function after ischemia better than placebo or mannitol.

Introduction

IN UROLOGIC PRACTICE, renal ischemia is frequently used during nephron-sparing procedures, such as laparoscopic and open partial nephrectomy. During these procedures, transient renal ischemia allows the tumor bed to be observed during tumor resection and reconstruction. The amount of time the human kidney can tolerate warm ischemia without substantial permanent injury is limited, however. Because clinical studies to establish thresholds for warm ischemia in humans are difficult to perform, the commonly accepted 30-minute upper time limit of warm ischemia is based on decades-old animal studies in the transplant literature.¹

Renal warm ischemia has recently become a salient issue in urologic practice with the introduction of laparoscopic partial nephrectomy. Although open surgery affords the surgeon the luxury of using ice slush for renal parenchymal cooling during ischemia, attempts at intrarenal and extrarenal parenchymal cooling during laparoscopic procedures have proved to be cumbersome, technically difficult, and expensive.

Currently, the only adjunct widely used to protect the kidney from ischemic reperfusion injury (IRI) is the administration of mannitol before renal artery clamping. The effectiveness of mannitol administration to protect against renal ischemia is based on urine flow and serum creatinine studies performed in an animal model.² Since this study was

published, however, a significant body of preclinical literature has demonstrated that erythropoietin (Epo) may protect organs from ischemia and the IRI that follows.³⁻⁹ To our knowledge, this study is the first to examine the impact of Epo on renal function after warm ischemia in a large animal model.

Methods

The protocol was approved by the Columbia University Institutional Animal Care and Use Committee. Thirty 20 to 25 kg class A dogs were allowed to acclimate for 2 days. Baseline basic metabolic profiles (BMP) and complete blood cell (CBC) counts were obtained during this time. All serum and urine samples were analyzed by Antech Diagnostic Laboratories (New York, NY).

Right laparoscopic nephrectomy

After acclimatization, all animals underwent a right laparoscopic nephrectomy. General anesthesia was induced with intravenous (IV) thiopental (10–17 mg/kg) and maintained with isoflurane (1%–5%) and oxygen (2–4 L/min). Once adequate general anesthesia was obtained and the animal's airway was secured, an IV catheter was placed and lactated Ringer solution was administered at 15 mL/kg/hr and increased as necessary to maintain stable vital signs during the course of the operation. Heart rate and rhythm, blood pressure, and pulse oximetry were monitored during the entire procedure.

Preoperatively, buprenorphine (0.03 mg/kg), cefazolin (30 mg/kg), and a fentanyl patch (50 µg/hr) were administered. The dogs were placed in the left lateral decubitus position and clipped, cleaned, and prepped using standard aseptic techniques. Pneumoperitoneum was established with Veress needle access, and after insufflation to 15 mm Hg, three 12-mm laparoscopic trocars were placed. The peritoneum was carefully incised over the area of the hilum, and the renal artery and vein were dissected free and divided with titanium clips and cold shears. Once the rest of the kidney was mobilized, the inferior trocar site was extended, and the specimen was extracted. All incisions were closed, and the animal recovered in the intensive care unit.

During the 2-week recovery period, animals received 3 days of postoperative cephalixin (30 mg/kg, po bid). Buprenorphine (0.03 mg/kg) was administered as needed for pain. After recovery, all animals were randomized to one of the following five groups:

- Group 1: Control surgery (anesthesia and 1.5 hours insufflation only) + IV physiologic saline (negative control) on initiation of the procedure.
- Group 2: Control surgery (anesthesia and 1.5 hours insufflation only) + IV Epo (500 units/kg) (positive control) on initiation of the procedure.
- Group 3: Ischemia + IV physiologic saline (given 30 minutes before 60 minutes of ischemia).
- Group 4: Ischemia + IV Epo (500 units/kg given 30 minutes before 60 minutes of ischemia).
- Group 5: Ischemia + IV mannitol (0.25 g/kg given 15 minutes before 60 minutes of ischemia).

Control surgery

Groups 1 and 2. Fourteen days after initial nephrectomy, the animals were returned to the operating suite. General

anesthesia was induced and maintained in a manner identical to the initial laparoscopic nephrectomy. A preoperative CBC was performed, and BMP sample was drawn. Once adequate general anesthesia was obtained and the animal's airway was secured, the animal was positioned in the right lateral decubitus position and padded, prepped, and draped in a manner identical to the first surgery.

Preoperatively, buprenorphine (0.03 mg/kg), cefazolin (30 mg/kg), and a fentanyl patch (50 µg/hr) were administered. Once Veress access was obtained and pneumoperitoneum was established, animals in group 1 received a 20 mL IV physiologic saline placebo injection, and animals in Group 2 received 500 units/kg of IV Epo. Abdominal insufflation was maintained at 15 mm Hg for 90 minutes. At the conclusion of 90 minutes of insufflation, the abdomen was decompressed, and the animal recovered in the intensive care unit.

Test surgery

Groups 3–5. Fourteen days after initial nephrectomy, the animals were returned to the operating suite. General anesthesia was induced and maintained in a manner identical to the initial laparoscopic nephrectomy. A pre-operative CBC was performed, and BMP sample was drawn. Once adequate general anesthesia was obtained and the animal's airway was secured, the animal was positioned in the right lateral decubitus position and padded, prepped, and draped in a manner identical to the first surgery.

Preoperatively, buprenorphine (0.03 mg/kg), cefazolin (30 mg/kg), and a fentanyl patch (50 µg/hr) were administered. The abdomen was insufflated to 15 mm Hg through Veress access, and three 12-mm laparoscopic trocars were deployed. At trocar placement, which was approximately 30 minutes before the implementation of ischemia, animals in group 3 received a 20 mL IV physiologic saline placebo injection, and animals in group 4 received 500 units/kg IV Epo injection. Animals in group 5 received 0.25 g/kg of IV mannitol approximately 15 minutes before ischemia.

Once the left renal hilum was identified and dissected free of its attachments, the renal artery was clamped with an atraumatic vascular bulldog clamp (Aesculap, Center Valley, PA) for 60 minutes. The renal vein was left patent in all cases. After 60 minutes of ischemic time, the vascular clamp was removed, the incisions were closed, and the animal recovered in the intensive care unit.

Recovery

Dogs in all groups survived for 28 days. During recovery, animals received 3 days of postoperative cephalixin (30 mg/kg, po bid). Buprenorphine (0.03 mg/kg) was administered as needed for pain. Twenty-four hour urine collections as well as a serum evaluation, including a CBC and BMP, were performed on postoperative days 1, 7, 14, and 21. Only blood was collected on day 28. Twenty-four hour urine collections were performed by collection of urine from the pan beneath each dog's cage. Dog food and water were double bowled to prevent spillage into the urine collection pan. No additional drugs or nephrotoxic agents were given during this time.

On postoperative day 28, general anesthesia was induced in all animals in a manner identical to that outlined above. Two open, excisional, corticomedullary renal biopsies were

TABLE 1. MEAN SERUM CREATININE LEVELS (MG/DL) BETWEEN THE GROUPS BEFORE AND AFTER WARM RENAL ISCHEMIA

	Group 1	Group 2	Group 3	Group 4	Group 5	P value
2 weeks postnephrectomy	1.02 ± 0.26	1.07 ± 0.15	0.98 ± 0.10	1.1 ± 0.08	1.1 ± 0.0	0.63
Peak after test/control surgery	1.18 ± 0.18	1.18 ± 0.10	1.52 ± 0.49	1.27 ± 0.14	1.32 ± 0.28	0.49
Sacrifice	1.07 ± 0.08	1.15 ± 0.19	1.15 ± 0.08	1.15 ± 0.10	1.13 ± 0.05	0.54

taken of the remaining renal unit, and the animal was sacrificed using a barbiturate overdose of 150 mg/kg according to American Veterinary Medical Association guidelines.

Urine and blood parameters were compared using the Kruskal-Wallis test.

Results

There were no intraoperative complications during any of the surgical procedures. The estimated blood loss for all procedures was less than 10 mL. Postoperatively, an area of fluctuation around a nephrectomy extraction incision developed in one animal (group 2), without evidence of fever or leukocytosis. This dog was presumptively treated for a possible abscess with 1 week of oral cephalexin, and the lesion resolved.

After initial nephrectomy and a 2-week recovery period, animals in all groups had statistically similar serum creatinine levels (Table 1). Similarly, after test and control surgeries, the peak level of serum creatinine and serum creatinine level at sacrifice did not significantly differ between groups. In addition, there was no statistically significant difference in baseline hematocrit or the peak hematocrit obtained after the second test/control surgery ($P = 0.07$ and 0.10 , respectively).

Although there were no statistically significant differences between the groups' serum parameters, there were significant differences between their urine parameters. Immediately after the second procedure, all dogs undergoing renal ischemia (groups 3–5) had significantly lower urine creatinine (Ucr) levels than those undergoing control insufflation (groups 1 and 2; $P < 0.01$). (Fig. 1 and Table 5) At the time of sacrifice, however, the group that received Epo before ischemia (group 4) had a 1.8- to 2.1-fold higher urine creatinine value than either the placebo physiologic saline injection group or mannitol group (groups 3 and 5, respectively).

A slightly different trend was seen with urine albumin (Ualb). Immediately after the second surgery, microalbumin-

uria was statistically similar in all groups ($P = 0.19$, Fig. 2 and Table 4). At the time of sacrifice, however, Ualb in the ischemia group receiving Epo (group 4) was much lower than either of the other ischemia groups (groups 3 and 5; $P < 0.0001$).

Discussion

End-organ ischemia can cause significant morbidity and even mortality. In certain instances, regulated, iatrogenic ischemia is necessary to accomplish surgical objectives. In urologic practice, warm ischemia is frequently applied during laparoscopic partial nephrectomy; however, the use of warm ischemia limits the time available for resection and reconstruction of the kidney.

Establishing a precise, safe, upper time limit that the human kidney can withstand renal ischemia is difficult to do without subjecting study participants to unacceptable risks. As such, based on information derived from the transplant literature, many urologists consider 30 minutes of warm ischemia to be the upper acceptable limit, although this remains a matter of heated debate.¹

The damage caused by warm ischemia is the end result of a complex series of processes. The majority of the damage, however, is not caused during the actual initial ischemic insult, but on reperfusion, a phenomenon known as IRI. IRI is a process that results in injury and morbidity in a myriad of disease processes, including coronary artery disease, stroke, and peripheral vascular disease. Before understanding the molecular mechanisms of IRI, scientists observed that short periods of sublethal ischemia could decrease and delay cell death during longer periods of ischemia.¹⁰ The concept of protecting tissues from IRI became known as ischemic preconditioning (IPC).

As the molecular mechanisms responsible for IRI were elucidated, it became evident that hypoxia inducible factor-1 (HIF-1) and its downstream targets are a pivotal component of the body's response to ischemia. HIF-1 is a heterodimer,

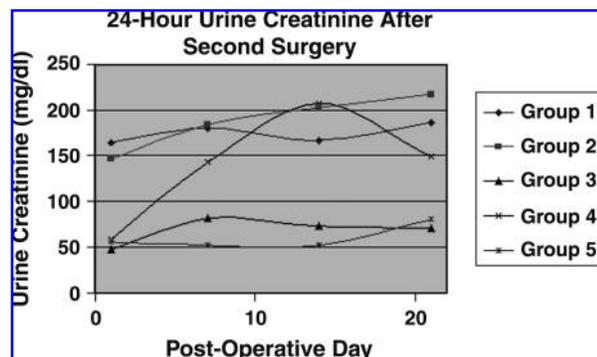


FIG. 1. Twenty-four hour urine creatinine after second surgery.

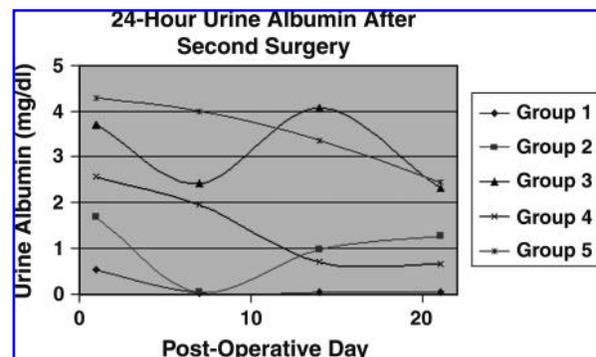


FIG. 2. Twenty-four hour urine albumin after second surgery.

TABLE 2. ERYTHROPOIETIN DOSES USED IN RODENT ISCHEMIA STUDIES

Authors	Year	Dose	Route	Epo formulation	Species
Malhotra ⁴	2006	2500 IU/kg	IP	Not specified	Wistar rats
Vaziri ⁵	1994	100 IU/kg*	IP	rhEpo	Sprague-Dawley rats
Yang ⁷	2003	3000 U/kg	IV	rhEpo	Sprague-Dawley rats
Cai ⁶	2003	5000 U/kg	IP	rhEpo	Sprague-Dawley rats
Sharples ³	2004	300 U/kg	IV	Not specified	Wistar rats

*Given once daily for 9 days.

IP = intraperitoneal; IV = intravenous; rhEpo = recombinant human erythropoietin.

composed of α and β subunits, that is found in almost all human tissues.¹¹ The HIF-1 β subunit is constitutively expressed.^{12,13} Production of the HIF-1 α subunit, however, is tightly regulated by oxygen tension.

In normoxic conditions, a conserved proline residue on the HIF-1 α subunit is hydroxylated by an oxygen-dependent mechanism that is closely associated to the von Hippel Lindau tumor suppressor. This hydroxylation targets the α subunit for ubiquitination and proteosomal degradation.^{14,15} During periods of hypoxia, the α subunit is not degraded, allowing HIF-1 to dimerize and bind to downstream targets.^{11,12} HIF-1 is a transcription factor with a basic DNA binding domain that recognizes the consensus sequence 5'-RCGTG-3'.¹⁶ Its downstream targets are varied and include genes involved in energy metabolism, angiogenesis, cell proliferation, erythropoiesis, and apoptosis.¹⁷⁻²⁰

One of the genes regulated by HIF-1 is erythropoietin. Epo is a 30.4kDa hematopoietic growth factor produced by the peritubular interstitial cells of the kidney.²¹ Under normal physiologic conditions, only minute quantities of Epo are present in the plasma,²² but during periods of hypoxia or anemia, the production of Epo is significantly upregulated.

In 1981, Prass and coworkers²³ demonstrated that brief periods of ischemia not only mimicked IPC, but also increased HIF-1 DNA binding and upregulated Epo transcription 7-fold. Inactivation of Epo using a soluble Epo receptor decreased the neuroprotective effects of this IPC by 40%. Using a rodent stroke model, Malhotra and colleagues⁴ demonstrated that intermittent hypoxia reduced infarct volume and induced the production of Epo and its receptor. The reduction in infarct volume conferred by IPC was largely reversed with administration of another, soluble, deactivating Epo receptor.

These findings remain consistent in other tissues subjected to ischemia. Semenza and associates⁶ showed that intermittent hypoxic IPC in the rat heart resulted in more than a five-fold increase in Epo mRNA expression with concomitant cardiac protection from ischemia. This increase in mRNA expression was evident in the kidney 1 hour after IPC. Plasma Epo levels rose 1 hour later.

In HIF-1 α -/- heterozygous knockout rats, this cardioprotection was lost, and Epo expression was not induced. Subsequently, when the rats were given Epo instead of being subjected to intermittent hypoxic IPC, results were similar to those animals who had undergone IPC: The heart regained more function after reperfusion with concomitant decrease in apoptosis and caspase-3 activity.

In 2003, Yang and colleagues⁷ published the first study using Epo to prevent IRI in the kidney. When compared with controls that did not receive any form of IPC, rats that

received Epo before ischemia had significantly lower serum creatinine levels 24 hours after ischemia, as well as less renal apoptosis, tubular necrosis, and red blood cell trapping. The following year, Sharples and coworkers³ demonstrated that Epo could confer protection against IRI when administered either 30 minutes or 5 minutes before ischemia or 5 minutes before reperfusion. Their data also supported Yang and associates' findings⁷ that Epo could ameliorate ischemia's effects on serum creatinine level, urine output, creatinine clearance, renal tubular architecture, and apoptosis.

To our knowledge, our study is the first study to demonstrate the effects of Epo on renal function after warm ischemia in a large animal model. The single renal unit model allows us to detect small differences in function that would be obscured by a normal contralateral kidney. We chose dogs for this study because the canine kidney's response to ischemia is more similar to the human response than other species commonly used, such as pigs.

In 2004 Baldwin and colleagues²⁴ demonstrated that after 90 minutes of warm ischemia, pigs with a solitary kidney regained normal renal function 7 to 14 days after the ischemic insult. These findings were duplicated by Laven and associates.²⁵ To define the upper limit of ischemia tolerated by pigs, Orvieto and coworkers²⁶ subjected pigs to 120 minutes of warm ischemia and found that only 2/3 of the animals had a 25% or greater decline in glomerular filtration rate by post-operative day 15. The mechanism that provides the porcine kidney with tolerance to ischemia is unclear, but the lack of irreversible renal damage after prolonged ischemia in the porcine kidney makes it unlike the human model and therefore unsuitable in this setting.

Dogs, however, are much more susceptible to irreversible renal damage. Lee and colleagues²⁷ demonstrated that, like humans, dogs with two kidneys subjected to 30 minutes of unilateral warm ischemia demonstrated no significant change in creatinine level or resistive index after 14 days. In 1992, Montanes and associates²⁸ demonstrated that 60 minutes of warm ischemia in a solitary canine kidney decreased canine

TABLE 3. RECOMBINANT HUMAN ERYTHROPOIETIN DOSES USED IN CANINE ISCHEMIA STUDIES

First Author	Year	Dose	Epo formulation	Route
Hirata ⁸	2006	1000 U/kg	rhEpo	IV
Hirata ⁹	2005	1000 U/kg	rhEpo	IV
Kawata ³⁰	2006	100 U/kg	rhEpo	IV
		5000 U/kg		

TABLE 4. MEAN URINE ALBUMIN LEVELS

	Group 1: Placebo, no ischemia	Group 2: Epo, no ischemia	Group 3: Placebo, ischemia	Group 4: Epo, ischemia	Group 5: Mannitol, ischemia	P value
Mean Ualb after second procedure (mg/dL)	0.53	1.68	3.70	2.56	4.3	0.19
Mean sacrifice Ualb (mg/dL)	0.05	1.28	2.32	0.65	2.43	0.0001

Ualb = urine albumin.

glomerular filtration rate by 22% after 48 hours. Sekhon and coworkers²⁹ demonstrated that 90 minutes of warm ischemia in a dog with a solitary kidney resulted in significant morbidity, a response that is more consistent with the human response.

The timing of drug administration was also based on previous experiments. In the seminal report on the administration of mannitol before renal ischemia, mannitol was most effective when given within 15 minutes of warm renal ischemia.² Similarly, for Epo, only one study has examined the effect of the timing of Epo administration on IRI: Sharples and coworkers³ administered a single IV Epo bolus either 30 minutes before ischemia, 5 minutes before reperfusion, or 30 minutes after reperfusion. Their results indicated that administration both before ischemia and before reperfusion have a prophylactic effect against IRI. Epo given after reperfusion did not demonstrate the same effects.

In our study, Epo was administered IV at a dose of 500 units/kg. In the rodent model, varying doses of recombinant human Epo (rhEpo) have been given using either intraperitoneal or IV injections. The dose and route of administration used in multiple rodent models are seen in Table 2. Doses ranged from repetitive doses of 100 units/kg to one-time boluses of 5000 units/kg. The different routes of administration make these dosing schema difficult to compare.

In dogs, there have been no studies examining the utility of Epo in a renal ischemia model; however, there have been several studies examining the effect of Epo on blood vessel formation and protection against ischemic damage in the myocardium and central nervous system.^{8,9,30} The doses of rhEpo used in canine studies are seen in Table 3. Doses from 100 units/kg up to 5000 units/kg have been tested and found to be effective in these settings. Because human epoetin alfa is not frequently given at doses higher than 500 units/kg, the animals in this study received 500 units/kg.

Our study demonstrates several things. First, it confirms that 60 minutes of warm ischemia causes renal damage

to the canine kidney that is still evident even after 1 month, making dogs a superior renal ischemia model to pigs. Second, despite the fact that significant postischemic renal function changes were detected by urine parameters, none of these alterations were detected by serum creatinine measurements, even in this solitary kidney model, which indicates that serum creatinine is not an adequately sensitive metric to discern renal function differences in this setting. The current body of literature that is based on serum creatinine measurements should therefore be reevaluated in this light.

Third, based on the results of the current study, it is evident that mannitol has little benefit when administered before ischemia, while Epo expedites recovery after an ischemic insult. Epo may therefore be a useful adjunct during procedures during which renal warm ischemia is applied, such as laparoscopic partial nephrectomy or donor nephrectomy.

There are some limitations to this study. Because most dogs would not tolerate Foley catheterization for more than a few hours, urine had to be collected from the under-cage pan, which is less accurate than continuous drainage. In addition, tests of urinary markers of renal function, such as neutrophil gelatinase associated lipocalin-2, are not performed by commercial laboratories, and commercially available antibodies are not compatible with canine antigens. As such, these parameters were not evaluated in this study.

In addition, Epo itself has some limitations. Although Epo did not cause polycythemia, or even a significant change in hematocrit, in any of our animals, it is currently contraindicated for use in patients with polycythemia. There have been reports that Epo administration may decrease the time to recurrence or promote the growth of Epo/Epo-receptor expressing tumors, such as breast cancer, cervical carcinoma, head and neck squamous-cell carcinomas, melanomas, neuroblastomas, and glioblastomas.³¹⁻³³ This effect, which is thought to be mediated through the Janus kinase and signal transducer and activator of transcription pathway, has not, however, been found to be a key element in the development or progression of renal-cell carcinoma.

TABLE 5. MEAN URINE CREATININE LEVELS

	Group 1: Placebo, no ischemia	Group 2: Epo, no ischemia	Group 3: Placebo, ischemia	Group 4: Epo, ischemia	Group 5: Mannitol, ischemia	P value
Mean Ucr after second procedure (mg/dL)	165.08	146.38	47.14	58.24	55.8	<0.01
Mean sacrifice Ucr (mg/dL)	186.26	216.47	70.66	149.05	80.68	0.01

Ucr = urine creatinine.

Conclusion

Despite the limitations of this study, these results demonstrate that ischemic preconditioning by IV Epo administration before renal ischemia may represent a significant improvement over the current standard-of-care, mannitol, in protecting the kidney from IRI. Clinical studies that evaluate the effectiveness of Epo in the prevention of ischemic damage to the kidney in the setting of laparoscopic partial nephrectomy are indicated before widespread clinical application.

Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

BMP = basic metabolic profile
CBC = complete blood cell
Epo = erythropoietin
HIF-1 = hypoxia inducible factor-1
IPC = ischemic preconditioning
IRI = ischemic reperfusion injury
IV = intravenous
rhEpo = recombinant human erythropoietin
Ualb = urine albumin
Ucr = urine creatinine

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