

# Effect of denervation and ischemia reperfusion injury on serum nitric oxide levels in rats

Umut Ozsoy, MSc, Ruya Mutluay, MD, Nezih Oygur, MD, Halide Akbas, MD, Sukru Sindel, MD, Muzaffer Sindel, PhD.

## ABSTRACT

**الأهداف:** تقييم أثر قطع الأعصاب للكلى، ومستوى مصل أكسيد النتريك، مع اختلاف فترة العلاج لإصابة إعادة التروية الافتقارية الكلوية.

**الطريقة:** تم تقسيم عدد 36 جرذاً ذكراً من نوع ويستار بشكل عشوائي إلى ستة مجموعات. خضعت جميع الجرذان لعملية استئصال الكلية اليمنى لخلق نموذج مفرد الكلية. تم قطع الأعصاب عن الكلية للجرذان وتعليقها لمدة 30-60 دقيقة. أجريت هذه الدراسة بجامعة أكدينس - بقسم التشريح - انطاليا - تركيا، خلال الفترة ما بين يونيو 2005م وحتى نوفمبر 2005م.

**النتائج:** سبب الأثر المتحد لقطع الأعصاب والإصابة الافتقارية زيادة ملحوظة في مستويات مصل أكسيد النتريك (NO) وانخفاض في (GFR).

**خاتمة:** تشير نتائجنا إلى أن قطع أعصاب الكلية لا يسبب فقط أي تغييرات في وظائف الكلى ولكن مع الإصابة الافتقارية يسوء الأمر ويؤدي إلى آثر ضارة لإصابة إعادة التروية الافتقارية، ويسبب أيضاً زيادة ملحوظة في مستويات مصل أكسيد النتريك (NO).

**Objectives:** To evaluate the effect of renal denervation and serum nitric oxide level with a different time course of renal ischemia-reperfusion injury.

**Methods:** Thirty-six male Wistar rats were randomized into 6 groups. All rats underwent right nephrectomy to create a single kidney model. Renal denervated and innervated rats were subjected to renal clamping for 30-60 minutes. The study was performed in the Department of Anatomy, Akdeniz University, Antalya, Turkey, between June and November 2005.

**Results:** Combined effect of denervation and ischemia may caused significant increase in serum nitric oxide levels and decrease in glomerular filtration rates.

**Conclusion:** Our results indicate that kidney denervation did not cause any changes in renal functions, but with ischemia it worsens the deleterious effect of ischemia-reperfusion injury, and causes a significant increase in serum nitric oxide levels.

*Saudi Med J 2008; Vol. 29 (11): 1561-1566*

*From the Departments of Anatomy (Ozsoy, Sindel), Surgery (Oygur), Biochemistry (Akbas), Faculty of Medicine, Akdeniz University, Antalya, and the Department of Nephrology (Mutluay, Sindel), Faculty of Medicine, Gazi University, Ankara, Turkey.*

*Received 13th July 2008. Accepted 15th October 2008.*

*Address correspondence and reprint request to: Dr. Muzaffer Sindel, Department of Anatomy, Faculty of Medicine, Akdeniz University, 07070 Antalya, Turkey. Tel. +90 (242) 2274485. Fax: +90 (242) 2274495. E-mail: sindelm@akdeniz.edu.tr*

Ischemia-reperfusion (IR) injury particularly that of the kidney, has been a topic of intensive study in the recent years because of its frequency and clinical significance.<sup>1</sup> Renal IR injury is pertinent to vascular and transplant surgery.<sup>2</sup> However, the pathogenesis of the injury has not been well defined. Oxygen free radicals due to ischemia may cause DNA scission and base modification, lipid peroxidation, or protein damage, and inactivation.<sup>1</sup> Mechanisms of IR injury have been considered including abnormalities in regional

**Disclosure.** This work was supported by grants from the Akdeniz University Scientific Research Projects Management Unit (2004.02.0122.14), Antalya, Turkey.

blood flow, endothelial/epithelial cell dysfunction, inflammation, and tubular obstruction.<sup>3</sup> Kidney nonfunction, delayed graft function, increased acute rejection, and late allograft dysfunction due to IR injury is the most important problem in transplantation.<sup>4</sup> The aim of the present study is to evaluate the effect of renal denervation and serum nitric oxide (NO) level with a different time course of renal IR injury in rats.

**Methods.** The Experimental Animal Committee at Akdeniz University approved the experimental protocols and animal care methods in the experiments. Male Wistar rats weighing 350-400gr were used in this study. Animals were housed in a light-controlled room with a 12-hours light/dark cycle and were allowed ad libitum access to food, water, and not fed a special diet that may affect NO levels. These rats were separated into 6 groups: Group I (n=6) underwent right nephrectomy, group II (n=6) underwent right nephrectomy and left renal ischemia by occlusion of the renal artery for 30 minutes, group III (n=6) underwent right nephrectomy and left renal ischemia by occlusion of the renal artery for 60 minutes, group IV (n=6) underwent right nephrectomy and left denervation, group V (n=6) underwent right nephrectomy, left denervation, and left renal ischemia by occlusion of the renal artery for 30 minutes, and group VI (n=6) underwent right nephrectomy, left denervation, and left renal ischemia by occlusion of the renal artery for 60 minutes (Table 1). To induce ischemic acute renal failure, rats were anesthetized with 10 mg/ kg xylazine hydrochloride (HCl) (Alfazyne 2%, Alfasan International Besloten Vennootschap [B.V.]), 50 mg/kg ketamine HCl (Alfamine 10%, Alfasan International B.V.), and the left kidney was exposed through an abdominal midline incision. The left renal artery and vein were occluded with a non-traumatic

clamp for 30 min or 60 min. At the end of the ischemic period, the clamp was released to allow reperfusion. Renal denervation was accomplished 5 min before the start of ischemia by cutting all visible nerves entering the renal hilus, stripping the renal artery and vein of adventitia, and applying a 10% phenol in 70% ethanol to the vessels.<sup>5</sup> Animals exposed to 30 min and 60 min ischemia were housed in metabolic cages at 24 hour after reperfusion. Venous blood samples were collected from the tail of all rats housed in metabolic cages at one-hour after reestablishment of blood flow. At the end of urine collection, blood samples were drawn from the thoracic aorta under 10mg/ kg xylazine HCl (Alfazyne 2%, Alfasan International B.V.), 50 mg/kg ketamine HCl (Alfamine 10%, Alfasan Int. B.V.) anesthesia. The study was performed in the Department of Anatomy, Akdeniz University between June and November 2005.

Blood samples were drawn from group I at one and 24 hour after nephrectomy, from group II, III, V and VI at one and 24 hours after reperfusion, from group IV at one and 24 hours after denervation. Twenty-four hours urine samples were collected by using metabolic cages. The blood samples were centrifuged (4000 rpm for 5 min) to separate serum. Serum and urine samples were stored at -80°C until analyses were performed. Serum creatinine, blood urea nitrogen (BUN) and urinary creatinine levels were measured by spectrophotometric methods on Modular PP analyzer (Roche Diagnostics, Basel, Switzerland). The results were expressed as mg/dL. Serum and urine electrolyte concentrations were measured on the same analyzer using ion-specific electrodes and internal reference solutions. The results were expressed as mEq/L. Serum NO levels were determined by using a commercial kit (Calbiochem, Catalog number: 482650). The assay involved the conversion of nitrate to nitrite by nitrate reductase. The detection of nitrite is determined as a colored azo-dye product of the Griess reaction that absorbs visible light. Serum samples containing significant levels of protein may produce a precipitate that may interfere with accurate measurement of NO. Therefore, we removed excess proteins by boiling 5 min and centrifuging 10,000 x 5 min, prior to performing the assay as mentioned in the kit insert. The concentration of NO was indirectly measured by determining both nitrate and nitrite levels in the same sample. The results were calculated as  $\mu\text{mol/L}$ . The glomerular filtration rates (GFR) were calculated by creatinine clearance. The results were expressed as mL/min. Fractional excretion of sodium (FeNa) was calculated using the formula:  $(\text{urinary sodium} \times \text{plasma creatinine}) / (\text{plasma sodium} \times \text{urinary creatinine}) \times 100$ . The results were expressed as percentage.

Statistical analysis was carried out using the SPSS package (version 11.0, SPSS, Chicago, Illinois, USA).

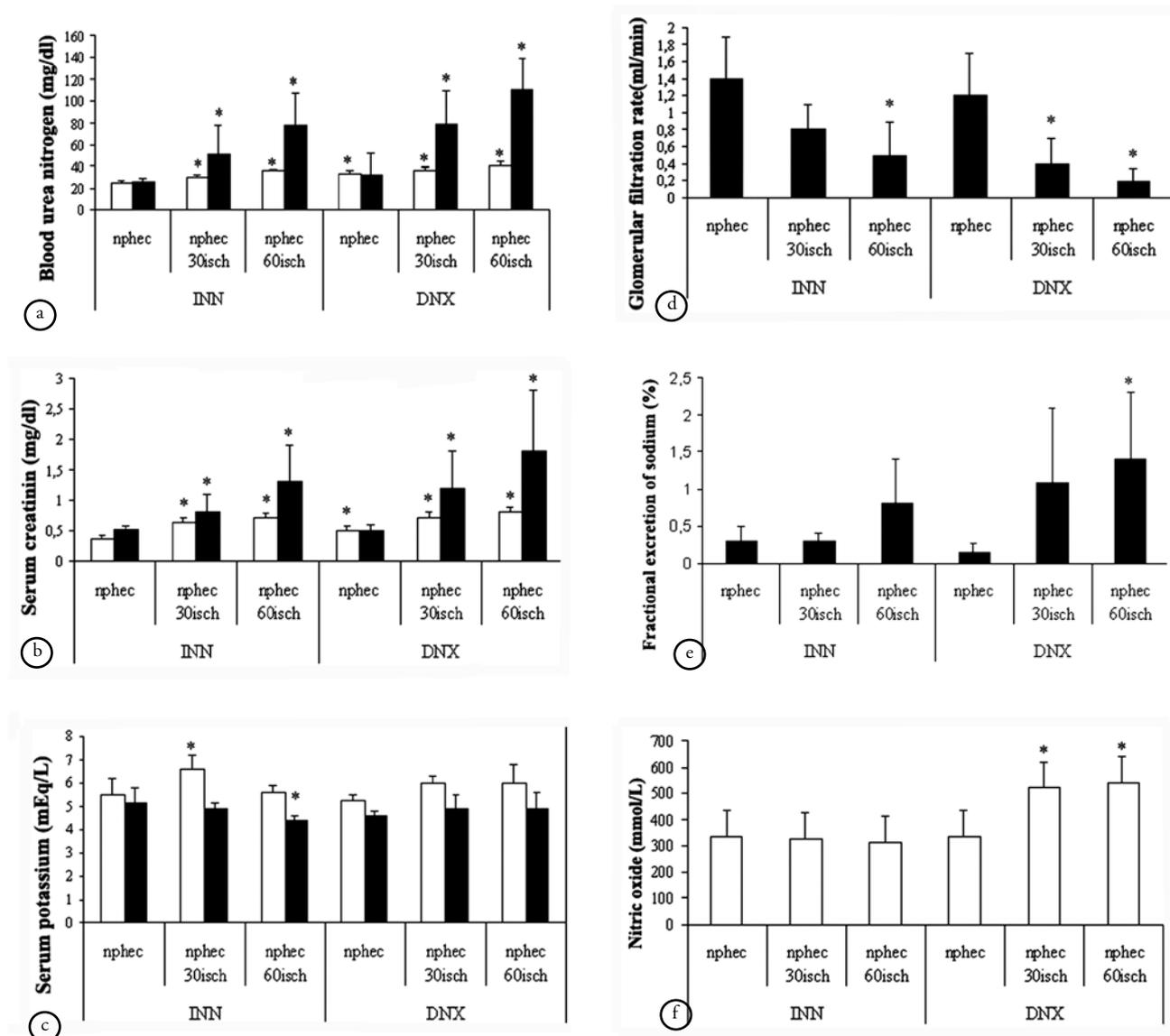
**Table 1** - List of experimental design.

Groups	Nephrectomy	Denervation	30 minute ischemia	60 minute ischemia
Group I	Applied	Not applied	Not applied	Not applied
Group II	Applied	Not applied	Applied	Not applied
Group III	Applied	Not applied	Not applied	Applied
Group IV	Applied	Applied	Not applied	Not applied
Group V	Applied	Applied	Applied	Not applied
Group VI	Applied	Applied	Not applied	Applied

Data were expressed as mean  $\pm$  standard deviation. A *p* value of  $<0.05$  was considered statistically significant. The Wilcoxon signed ranks test and the Mann–Whitney U test were used to evaluate differences between the groups.

**Results.** Effects of 30 and 60 min IR injury on renal function are summarized in Figures 1a-1f. Renal functions of rats, subjected to 30-min ischemia (group II) were measured at one and 24 hours after the reperfusion. As compared with sham-operated rats (group I), group II rats showed a significant increase in serum creatinine and BUN concentrations. Ischemia-reperfusion injury

did not significantly influence the glomerular filtration rates in this group. On the other hand, a marked deterioration was observed with 60-min ischemia in group III rats. In this group, serum creatinine and BUN levels were found as significantly increased while the GFR values were found as decreased. At the end of the first-hour, there was no significant alteration at serum NO levels in both groups when compared with sham operated group. Combined effects of denervation and ischemia on renal function were evaluated. Renal function of rats subjected to 30-min ischemia showed a marked deterioration when measured at one and 24 hours after the reperfusion. As compared with sham-



**Figure 1** - Effects of renal denervation and ischemia reperfusion injury on a) blood urea nitrogen, b) serum creatinine, c) serum potassium, d) glomerular filtration rate, e) fractional excretion of sodium, f) and nitric oxide at one hour after reperfusion (open bars) and at 24 hours after reperfusion (close bars) . \**p* < 0.05: statistically significant versus INN nphec group, INN - innervation, DNX - denervation, nphec - nephrectomy, nphec 30isch - nephrectomy and 30 minute ischemia, nphec 60isch - nephrectomy and 60 minute ischemia.

operated rats, this group showed significant increases in blood urea nitrogen, serum creatinine concentration, and significant decreases in GFR. Extending the ischemia time to 60 minutes worsened the renal function of rats when measured at one and 24 hours after the reperfusion. As compared with sham-operated rats, denervated acute renal failure rats showed significant increases in blood urea nitrogen, serum creatinine concentration, and significant decreases in GFR. In both groups serum NO significant higher than sham operated group and Fena values were over one. When innerve and denervated groups with the same ischemia periods were compared with each other, denervated group showed a slight increase in blood urea nitrogen, serum creatinine concentration, and a slight decrease in GFR, but it was not significant.

**Discussion.** Ischemic acute renal failure is a frequent clinical syndrome with high morbidity and mortality.<sup>6</sup> Reperfusion of previously ischemic renal tissue initiates a complex cellular event that results in injury and the eventual death of renal cells due to a combination of apoptosis and necrosis.<sup>7</sup> The molecular mechanisms underlying the ischemia/reperfusion-induced renal injury are poorly understood, but it has been reported that several causal factors (ATP depletion, reactive oxygen species, phospholipase activation, neutrophil infiltration, vasoactive peptides, and so forth) are contributive to the pathogenesis of this renal damage.<sup>8</sup> This damage is related with the time course of the ischemia. Reports have cited 30 minutes as the maximum tolerable limit of renal ischemia time.<sup>9-12</sup> Williams *et al*,<sup>13</sup> reported that a renal ischemia of 45 min and Paller and Hebbel<sup>14</sup> reported a renal ischemia of 60 min significantly improved renal damage in the rat kidney. Paller and Hebbel<sup>14</sup> and Paller *et al*<sup>15</sup> reported that immunohistochemical and biochemical damage appears after 4 hours, and it peaks at 24 hours. The standard methodology of acute surgical /chemical renal denervation is at least one hour recovery must be allowed after maneuver.<sup>16</sup> In the present study, we applied 30-min, and 60 min ischemia and the function of kidney were assessed on the first and 24th hours. All rats underwent right nephrectomy to create a single kidney model. Renal function of rats subjected to 30-min ischemia measured on the first and 24 hours after the reperfusion. Acute renal failure rats showed significant increases in BUN and serum creatinine concentration. In this group, there was no significant difference in GFR. The 60 minute groups showed a marked deterioration when measured one and 24 hours after the reperfusion. In the 60 minute groups BUN and serum creatinine significantly increased. Extending the ischemia period from 30min to 60min caused significant decreases in GFR. The renal circulation,

tubular reabsorption, and release of renin are under multiple control by the renal nerves, hormones, and paracrine active agents.<sup>17</sup> In the kidney, increased renal sympathetic nerve activity regulates the functions of the intrarenal effectors: the tubules, the blood vessels, and the juxtaglomerular granular cells. This enables a physiologically appropriate coordination between the circulatory, filtration, reabsorptive, excretory, and renin secretory contributions to overall renal function.<sup>18</sup> Removal of the neural input by renal denervation, leading to inhibition of tubular transport (denervation natriuresis) and, under some circumstances, to a decrease in renin secretion and an increase in renal blood flow (RBF) may be expected to be compensated, at least in part, by control systems working in the opposite direction. For instance, humoral vasoconstrictor agents could help offset the loss of vasoconstrictor tone provided by noradrenaline released from renal nerve endings. However, it is possible that some of the compensatory mechanisms do not come into play immediately after denervation, but require some time to develop.<sup>19</sup> In our study, we observed significant increments in serum creatinine and BUN levels in denervated group when compared with sham operated group 24 hours after the reperfusion. However, we did not found any significant difference between the other measured parameters. These results suggest that kidney denervation without ischemia did not cause any changes in renal functions. Ogawa *et al*<sup>20</sup> demonstrated that renal denervation abolishes the protective effects of ischemic preconditioning on function and hemodynamics in ischemia-reperused rat kidneys. This results are partly parallel with our findings because of the importance of renal innervation. In the current study renal function of denervated rats subjected to 30-min ischemia showed a marked deterioration more than innervated rats and the tolerable ischemia time of the innervated kidneys was significantly longer than denervated kidneys. After nephrectomy, the kidney undergoes both hemodynamic and structural changes. The hemodynamic changes involve increased RBF and decreased renal vascular resistance. The structural adaptation to unilateral nephrectomy is hypertrophy of the remaining kidney.<sup>21</sup> Nitric oxide plays an important role in eliminating the hemodynamic changes caused by nephrectomy.<sup>22</sup> Activation of NO may function as an inhibitory neurotransmitter, regulating the activation of renal mechanosensory nerve fibers.<sup>23</sup> Reduction of renal mass by unilateral nephrectomy results in an immediate increase in RBF to the remnant kidney, followed by compensatory renal hypertrophy. Whether the increase in RBF after unilateral nephrectomy is mediated by NO.<sup>23,24</sup> Suprarenal aortic clamping and reperfusion increase medullary and cortical NO synthesis.<sup>25</sup> Tissue NO levels increase dramatically during ischemia an effect that has been shown to be

partially independent of NO synthases.<sup>26,27</sup> Saito and Miyagawa<sup>28</sup> measured blood flow and NO release in the rat kidney by real-time monitoring. They reported that immediately following the clipping of the renal artery, NO release rapidly and increased after removing the clip; NO release immediately and returned to three-quarters of the basal level. We assessed the renal functions at one and 24 hours and NO at one hour in order to measure the NO levels early period of this fall. In the present study, serum NO levels were measured one hour after the reperfusion. In contrast to the sham operated rats in the renal denervated rats, subjected to 30 and 60-min ischemia, NO levels were significantly higher. In innervated group, there were no significant differences with sham operated rats within 60 minutes (Figure 1f). However, in denervated group serum NO levels significantly increased. In this group, it has been suggested that NO synthases continue during reperfusion. Our results show that at physiological conditions denervation does not affect renal functions, but it is worsening the injurious effect of IR injury. The question raised is whether such functionally specific fiber groups existed in the renal sympathetic innervation that could provide selective and specific innervation and control of the 3 renal neuro effectors: the vasculature, the tubules, and the juxtaglomerular granular cells.<sup>29</sup> Future studies need to be designed to address these still unsolved issues. In addition, some groups have suggested that the use of serum creatinine as a single marker of renal function is suboptimal in a research or clinical setting.<sup>30,31</sup> Thus, there is a paucity of clinical or experimental series that have adequately examined renal function following prolonged ischemic insults.<sup>11</sup> Consequently, it is expected that new analysis methods and parameters should be developed for examining renal functions.

## References

- Plestina S, Gamulin S. Kidney ischaemia-reperfusion injury and polyribosome structure. *Nephron* 2001; 89: 201-207.
- Weight SC, Furness PN, Nicholson ML. New model of renal warm ischaemia-reperfusion injury for comparative functional, morphological and pathophysiological studies. *Br J Surg* 1998; 85: 1669-1673.
- Yang B, Jain S, Pawluczyk IZ, Imtiaz S, Bowley L, Ashra SY, et al. Inflammation and caspase activation in long-term renal ischemia/reperfusion injury and immunosuppression in rats. *Kidney Int* 2005; 68: 2050-2067.
- Döşlütöğlü HH, Aktan AO, Yeğen C, Okboy N, Yalçın AS, Yahn R, et al. The cytoprotective effects of verapamil and iloprost (ZK 36374) on ischemia/reperfusion injury of kidneys. *Transpl Int* 1993; 6: 138-142.
- Fujii T, Kurata H, Takaoka M, Muraoka T, Fujisawa Y, Shokoji T, et al. The role of renal sympathetic nervous system in the pathogenesis of ischemic acute renal failure. *Eur J Pharmacol* 2003; 481: 241-248.
- Thadhani R, Pascual M, Bonventre JV. Acute renal failure. *N Engl J Med* 1996; 334: 1448-1460.
- Lieberthal W, Levine JS. Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. *Am J Physiol* 1996; 271 (3 Pt 2): F477-F488.
- Edelstein CL, Ling H, Schrier RW. The nature of renal cell injury. *Kidney Int* 1997; 51: 1341-1351.
- Desai MM, Gill IS, Ramani AP, Spaliviero M, Rybicki L, Kaouk JH. The impact of warm ischaemia on renal function after laparoscopic partial nephrectomy. *BJU Int* 2005; 95: 377-383.
- Islam CF, Mathie RT, Dinneen MD, Kiely EA, Peters AM, Grace PA. Ischaemia-reperfusion injury in the rat kidney: the effect of preconditioning. *Br J Urol* 1997; 79: 842-847.
- Laven BA, Orvieto MA, Chuang MS, Ritch CR, Murray P, Harland RC, et al. Renal tolerance to prolonged warm ischemia time in a laparoscopic versus open surgery porcine model. *J Urol* 2004; 172 (6 Pt 1): 2471-2474.
- Novick AC. Surgery of the kidney. In: Walsh PC, Retik AB, Vaughan ED Jr, Wein AJ, editors. *Campbell's Urology*. 8th ed. Philadelphia (PA): W. B. Saunders Co; 2002. p. 3570-3643.
- Williams P, Lopez H, Britt D, Chan C, Ezrin A, Hottendorf R. Characterization of renal ischemia-reperfusion injury in rats. *J Pharmacol Toxicol Methods* 1997; 37: 1-7.
- Paller MS, Hebbel RP. Ethane production as a measure of lipid peroxidation after renal ischemia. *Am J Physiol* 1986; 251 (5 Pt 2): F839-F843.
- Paller MS, Hoidal JR, Ferris TE. Oxygen free radicals in ischemic acute renal failure in the rat. *J Clin Invest* 1984; 74: 1156-1164.
- Walkowska A, Kompanowska-Jeziarska E, Sadowski J. Nitric oxide and renal nerves: comparison of effects on renal circulation and sodium excretion in anesthetized rats. *Kidney Int* 2004; 66: 705-712.
- DiBona GF, Kopp UC. Neural control of renal function. *Physiol Rev* 1997; 77: 175-197.
- DiBona GF. Neural control of the kidney: functionally specific renal sympathetic nerve fibers. *Am J Physiol Regul Integr Comp Physiol* 2000; 279: R1517-R1524.
- Kompanowska-Jeziarska E, Walkowska A, Johns EJ, Sadowski J. Early effects of renal denervation in the anaesthetised rat: natriuresis and increased cortical blood flow. *J Physiol* 2001; 531 (Pt 2): 527-534.
- Ogawa T, Mimura Y, Kaminishi M. Renal denervation abolishes the protective effects of ischaemic preconditioning on function and haemodynamics in ischaemia-reperfused rat kidneys. *Acta Physiol Scand* 2002; 174: 291-297.
- Brenner BM. Nephron adaptation to renal injury or ablation. *Am J Physiol* 1985; 249 (3 Pt 2): F324-F337.
- Lahera V, Salom MG, Miranda-Guardiola F, Moncada S, Romero JC. Effects of NG-nitro-L-arginine methyl ester on renal function and blood pressure. *Am J Physiol* 1991; 261 (6 Pt 2): F1033-F1037.
- Kopp UC, Cicha MZ, Smith LA, Hökfelt T. Nitric oxide modulates renal sensory nerve fibers by mechanisms related to substance P receptor activation. *Am J Physiol Regul Integr Comp Physiol* 2001; 281: R279-R290.
- Sigmon DH, Gonzalez-Feldman E, Cavin MA, Potter DL, Beierwaltes WH. Role of nitric oxide in the renal hemodynamic response to unilateral nephrectomy. *J Am Soc Nephrol* 2004; 15: 1413-1420.
- Myers SI, Wang L, Liu F, Bartula LL. Suprarenal aortic clamping and reperfusion decreases medullary and cortical blood flow by decreased endogenous renal nitric oxide and PGE2 synthesis. *J Vasc Surg* 2005; 42: 524-531.

26. Aragno M, Cutrin JC, Mastrocola R, Perrelli MG, Restivo F, Poli G, et al. Oxidative stress and kidney dysfunction due to ischemia/reperfusion in rat: attenuation by dehydroepiandrosterone. *Kidney Int* 2003; 64: 836-843.
27. Salom MG, Arregui B, Carbonell LF, Ruiz F, González-Mora JL, Fenoy FJ. Renal ischemia induces an increase in nitric oxide levels from tissue stores. *Am J Physiol Regul Integr Comp Physiol* 2005; 289: R1459-R1466.
28. Saito M, Miyagawa I. Real-time monitoring of nitric oxide in ischemia-reperfusion rat kidney. *Urol Res* 2000; 28: 141-146.
29. DiBona GF. Neural control of the kidney: past, present, and future. *Hypertension* 2003; 41 (3 Pt 2): 621-624.
30. Ward JP. Determination of the Optimum temperature for regional renal hypothermia during temporary renal ischaemia. *Br J Urol* 1975; 47: 17-24.
31. Shemesh O, Golbetz H, Kriss JP, Myers BD. Limitations of creatinine as a filtration marker in glomerulopathic patients. *Kidney Int* 1985; 28: 830-838.

#### Related topics

Shackebaei D, Godini A, Reshadat S. The effects of diazepam in cardio depressant concentration on the function of isolated rat heart in ischemia-reperfusion. *Saudi Med J* 2008; 29: 847-853.

Ao Y, Peng RX, Yang J, Wang BH, Falck JR, Dan H, et al. Expression of epoxygenases belonging to CYP2 family in rat myocardial ischemia/reperfusion injury in vivo. *Saudi Med J* 2008; 29: 23-29.

Kavakli A, Sahna E, Parlakpınar H, Yahsi S, Ogeturk M, Acet A. The effects of melatonin on focal cerebral ischemia-reperfusion model. *Saudi Med J* 2004; 25: 1751-1752.