

# The creation of unilateral intermittent and unintermittent renal ischemia-reperfusion models in rats

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## Abstract

**Background and Aim:** This study aims to establish unilateral intermittent and unintermittent partial nephrectomy-like renal ischemia-reperfusion (I-R) model in rats and to compare the results with biochemical findings.

**Material and Methods:** The study was conducted on 24 adult 8-week-old male Wistar-Albino rats, each weighing s200–250 g. The rats were divided into three groups. In the Sham group ( $n = 8$ ), the kidney was surgically exposed and closed. We designed experimental I-R models in the second group ( $n = 8$ , a total of 30-min ischemia model in the manner of 3 intermittent sets 8 minutes clamping and 2 min unclamping) and in the third group ( $n = 8$ , one session of 30-min unintermittent ischemia). In postoperative day 1, the rats were sacrificed, and the effects of I-R models on the renal tissue were comparatively assessed by evaluating serum Neutrophil Gelatinase-Associated Lipocalin (NGAL), serum kidney injury molecule-1 (KIM-1), urinary NGAL, urinary KIM-1, and serum creatinine levels.

**Results:** Urinary NGAL and KIM-1 levels were significantly higher in the continuous ischemia group when compared to those in the sham and intermittent ischemia groups ( $P < 0.05$ ). In the intermittent ischemia group, urinary NGAL and urinary KIM-1 levels were significantly higher than those in the sham group ( $P < 0.05$ ). Although the results of serum NGAL, serum KIM-1, and serum creatinine levels seemed to be in parallel to the results of urinary markers, no statistically significant difference was found.

**Conclusion:** Renal injury was significantly less in the intermittent I-R model when compared to that in the unintermittent I-R model in our experimental rat study.

**Keywords:** Intermittent ischemia-reperfusion, ischemia-reperfusion injury, partial nephrectomy, rats, renal failure

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## INTRODUCTION

Renal tumors have been more frequently diagnosed and treated at earlier stages due to improvements in the imaging modalities in the last decades. Although radical

nephrectomy was the gold-standard in surgical treatment of renal tumors in the past, today, nephron-sparing surgery has become a widely accepted surgical method in the treatment of renal tumors as a result of specific facts such

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as the increased risk of development of hypertension and chronic renal failure in the patients with solitary kidney, data indicating similar oncological outcomes in the radical and partial nephrectomy patients, availability of detailed anatomical knowledge about the tumoral and vascular structures of the kidney in the last two decades, and a raised awareness toward the functional role of the residual parenchymal tissue of the involved kidney. However, ischemia-reperfusion (I-R) injury resulting from transient blockage of blood flow in the renal artery during partial nephrectomy operations is still a real problem.

Surgical techniques without vascular clamping that are aimed to avoid ischemia have brought about a higher amount of blood loss and a higher rate of complications.<sup>[1]</sup> “Selective-super-selective arterial clamping technique” has been proposed to minimize and limit renal ischemia in an attempt to take the available methods further, and the only endeavor was to control vascular structure supplying the tumor.<sup>[2,3]</sup> Although this technique is associated with less global renal ischemia, it has limited use, and it has not been put into practice except for a few centers due to practical difficulties in medial and hilar tumors, high number of variations in vascular anatomy of the kidney, injury to the other vascular and parenchymal structures during selective devascularization, aberrant vascularization in some tumors, and a steep learning curve.<sup>[4,5]</sup> Anatomical variations of the renal arterial vasculature have further narrowed this field.

According to the current knowledge, intracellular overproduction of free oxygen radicals (FORs) following reperfusion and the resulting lipid peroxidation is the main trigger initiating apoptosis and tissue damage in the I-R damage. Cytokines produced at the tissue level (e.g., tumor necrosis factor- $\alpha$ , interleukin-6) initiate leukocyte accumulation, and intracellular JAK/STAT activation plays a role in the apoptotic process.<sup>[6,7]</sup> Several agents, including doxycycline, leptin, levosimendan, ascorbate, and iloprost, have been experimented to stop and prevent this process, all of which have not found a place in daily practice, although their benefits have been demonstrated.<sup>[8-12]</sup>

Today, we have arrived at a point where the clamping of the renal artery is not routinely used except for small tumors with peripheral localization, and the I-R damage mentioned above has not been precluded.

The present study aims to perform intermittent clamping of the renal artery in a rat model with certain intervals instead of unintermittent clamping (30 min) and expose the kidney to ischemia of shorter duration (8 min clamping-2 min unclamping), and thus to evaluate whether this

causes less extensive kidney damage in a controlled and comparative study.

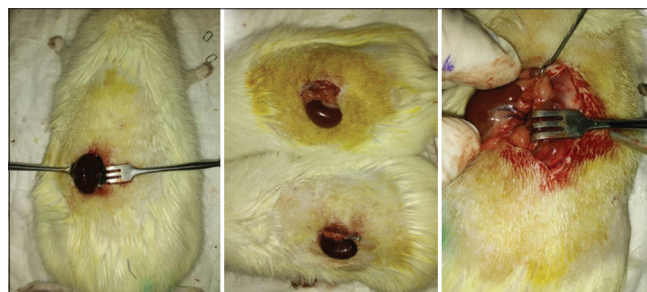
## MATERIALS AND METHODS

This experimental animal study was conducted in İstanbul Medipol University Medical Research Centre. An ethics approval was granted (March 17, 2017-Decree no: 11) from İstanbul Medipol University Animal Experiments Local Ethics Committee, and the experimental animals were obtained from İstanbul Medipol University Medical Research Centre. Twenty-four adult (8-week-old) male Wistar-Albino rats, each weighing 200–250 g, were used in the study. The power analysis of the study was conducted by evaluating similar studies in the literature, and it was determined that the inclusion of ideally eight rats would be sufficient. The rats were maintained in dry cages for acclimatization to the laboratory environment before the experiment (22°C 2, 12 h light/12 h dark cycle, 1-atmosphere pressure). They were given standard rat bated and tap water.

Standard surgical instruments were used in operation. The anesthesia was produced by intraperitoneal administration of ketamine 75 mg/kg and xylazine 10 mg/kg. Following the surgical procedure, the rats were given care in their cages for one day, and they were maintained in the laboratory environment. The subjects that completed all study procedures, including the operation and follow-up period, were sacrificed at 24 h after surgery.

A total of 24 Wistar-Albino rats were randomized into three groups.

**Group 1 (Sham Operation Group):** The back of rats was shaved, disinfected with 70% ethyl alcohol, and a left vertical flank incision was used to enter the skin. The left kidney was explored, and the renal artery was located [Figure 1]. After ensuring that the subject was sufficiently exposed to surgical and anesthetic stress, the operation was completed without performing further procedures by



**Figure 1:** Dorsal-vertical incision, microaneurysm clamps, and vascular structures

closing the incision with a 4-0 polyglactin suture, and the subjects were left to recover.

**Group 2 (Intermittent Ischemia Group):** The back of rats was disinfected with 70% ethyl alcohol after shaving, and a left vertical flank incision was used to enter the skin. The left kidney was explored, and the renal artery was located. The left renal artery was then isolated, and the subjects were exposed to 30 min of intermittent ischemia (three sets of I-R with 8 min of clamping followed by 2 min of unclamping) using atraumatic microaneurysm clamps. The procedure was then completed by closing the skin incision with 4-0 polyglactin sutures, and the subjects were left to recover.

**Group 3 (Unintermittent Ischemia Group):** The back of rats was disinfected with 70% ethyl alcohol after shaving, and a left vertical flank incision was used to enter the skin. The left kidney was explored, and the renal artery was located. The left renal artery was isolated, and the subjects were exposed to 30 min of unintermittent ischemia followed by reperfusion using atraumatic microaneurysm clamps. The procedure was then completed by closing the skin incision with 4-0 polyglactin sutures, and the subjects were left to recover.

Serum Neutrophil Gelatinase-Associated Lipocalin (NGAL), serum kidney injury molecule-1 (KIM-1), urinary NGAL, urinary KIM-1, and serum creatinine were used as markers of tissue damage. NGAL and KIM-1 were evaluated using an enzyme-linked immunosorbent assay, and serum creatinine level was measured using the Jaffe's method.

Descriptive statistics included mean, standard deviation, median (minimum-maximum), frequency, and ratio. A Kolmogorov–Smirnov test was used to evaluate the distribution of variables. The analysis of variance and the Kruskal–Wallis test were used in the analysis of quantitative variables. SPSS 22.0 software package was used in statistical analysis.

## RESULTS

Descriptive and comparative statistics for the markers of tissue damage are shown in Table 1 and Figure 2.

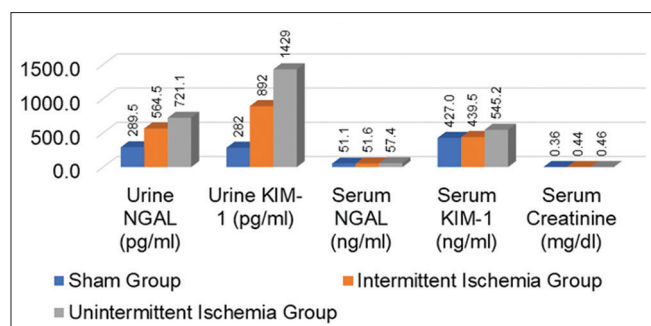
Urinary NGAL was significantly higher in the unintermittent ischemia group than in the intermittent ischemia and sham operation groups ( $P > 0.05$ ). Urinary NGAL was significantly higher in the intermittent ischemia group than in the sham operation group ( $P > 0.05$ ). Urinary KIM-1 level was significantly higher in the unintermittent ischemia group than in the intermittent ischemia and sham operation groups ( $P > 0.05$ ). The urinary KIM-1 level was significantly higher in the intermittent ischemia group than in the sham operation group ( $P > 0.05$ ).

Serum NGAL did not significantly differ among the unintermittent ischemia, intermittent ischemia, and sham operation groups ( $P > 0.05$ ). Serum KIM-1 did not significantly differ among the unintermittent ischemia, intermittent ischemia, and sham operation groups ( $P > 0.05$ ). Serum creatinine did not significantly differ among the unintermittent ischemia, intermittent ischemia, and sham operation groups ( $P > 0.05$ ).

**Table 1: Descriptive and comparative statistics of serum and urinary results in groups**

	Minimum-Maximum	Median	Mean±SD	P
Urinary NGAL (pg/ml)				
Sham group	203-547	269	289.5±110.6* <sup>†</sup>	0.000 (A)
Intermittent ischemia group	391-703	564	564.5±106.0*	
Unintermittent ischemia group	668-772	721	721.1±31.7	
Urinary KIM-1 (pg/ml)				
Sham group	148-369	282	282.1±72.6* <sup>†</sup>	0.000 (A)
Intermittent ischemia group	537-1267	891	891.5±232.6*	
Unintermittent ischemia group	891-2410	1429	1429.3±450.2	
Serum NGAL (ng/ml)				
Sham group	35-69	51	51.1±10.6	0.119 (K)
Intermittent ischemia group	46-54	53	51.6±3.4	
Unintermittent ischemia group	50-66	57	57.4±5.5	
Serum KIM-1 (ng/ml)				
Sham group	388-470	425	427.0±27.8	0.053 (K)
Intermittent ischemia group	325-543	416	439.5±82.0	
Unintermittent ischemia group	397-870	511	545.2±146.2	
Serum creatinine (mg/dl)				
Sham group	0.28-0.52	0,33	0.36±0.09	0.731 (K)
Intermittent ischemia group	0.30-0.93	0,36	0.44±0.21	
Unintermittent ischemia group	0.23-1.33	0,36	0.46±0.36	

\*Difference with unintermittent ischemia group,  $P < 0.05$ , <sup>†</sup>Difference with intermittent ischemia group  $P < 0.05$ . ANOVA: Analysis of variance, A: ANOVA (Tukey test), K: Kruskal-wallis, NGAL: Neutrophil gelatinase-associated lipocalin, KIM-1: Kidney injury molecule-1, SD: Standard deviation



**Figure 2:** Graphical comparison of urinary neutrophil gelatinase-associated lipocalin, kidney injury molecule-1 and serum neutrophil gelatinase-associated Lipocalin, kidney injury molecule-1, C-reatinine levels in groups

## DISCUSSION

Partial nephrectomy is a commonly used surgical method in the treatment of kidney tumors, resulting in satisfactory outcomes. Open surgery was the preferred technique in years after it was first introduced, but open surgery has been successfully replaced by laparoscopic and robotic surgery in recent years. Such new methods have found a place in the practice due to satisfactory oncological outcomes. However, endoscopic operations do not fulfill the conditions of cold ischemia, and the patient is often exposed to warm ischemia, the duration of which cannot be minimized as in open surgery. Despite all favorable factors, warm ischemia during surgery has remained a significant problem for long years. Initially, a mean warm ischemia period of 30–45 min was criticized, but it was later well established that even minimal ischemia damages the kidney and induces an apoptotic process through toxic and immunological injury following reperfusion.<sup>[6,7]</sup>

Several studies have been conducted to demonstrate I-R injury in the kidney, and a large number of markers have been described. Some of these markers are those used to indicate tubular injury, and other markers include FORs.

More than two hundred markers have been described, which indicate tubular injury, and more extensive research has been conducted into 25 to 30 of these markers.<sup>[13]</sup> Today we have arrived at a point where NGAL, KIM-1, Cystatin C, and L-FABP have been established as the most commonly used markers alone or in combination with each other yielding the highest sensitivity and specificity to determine kidney damage.

There is no readily available method of treatment for I-R damage. Several agents, including doxycycline, leptin, levosimendan, ascorbate, and iloprost, have been attempted and used with success, although they have not

found a place in daily practice.<sup>[8-12]</sup> Liu *et al.* have made a trial of an off-clamp technique to avoid such damage; however, this approach has failed due to excessive blood loss, postoperative urine leakage, and residual tumor tissue in tumors suitable for partial nephrectomy but which are large, close to the renal hilus and adjacent to the collecting system, and this has necessitated temporary clamping of renal vascular structures.<sup>[14]</sup> Gill *et al.* have carried a step forward regarding arterial clamping in the available methods by describing “selective-super-selective arterial clamping technique” in order to minimize and limit renal ischemia and endeavored to control only vascular structures supplying the tumor.<sup>[3]</sup> A high rate of variation in renal arterial anatomy has further narrowed this field. Nguyen and Gill suggested early declamping of the renal artery and controlling the parenchyma and collecting system with continuous medullary stitches and performing capsular repair without exposure to ischemia. However, their technique has found limited practice due to excessive blood loss.<sup>[15]</sup> Today’s practice has arrived at a point where the clamping of the renal artery is not routinely used in our country as well as in the world, except for small tumors with peripheral localization, and the I-R damage mentioned above has not been precluded.

The concepts of “ischemic preconditioning” and “ischemic postconditioning” have been suggested to minimize the effects of I-R damage on the tissues and organs and increase tissue strength.<sup>[16]</sup> The aim in ischemic preconditioning is to expose the organ that will potentially be exposed to I-R injury (donor kidney, or kidney and liver that will be exposed to ischemia during cardiac surgery) to short-term intermittent ischemic attacks before the anticipated ischemic period, and thus precondition the organ to prolonged ischemia period.<sup>[17]</sup> Ischemic postconditioning is based on controlled and intermittent reperfusion instead of rapid reperfusion of the tissues that have been exposed to ischemia (as in cerebral ischemia), and the studies have demonstrated less extensive tissue damage.<sup>[18]</sup> Although the underlying pathophysiological mechanism has not been fully understood, it is claimed that tissue damage is less extensive due to decreased expression of adhesion molecules that emerge at the tissue level during reperfusion and mediate tissue damage.<sup>[19]</sup> In the present study, intermittent reperfusion of the kidney during partial nephrectomy instead of exposing the kidney to a prolonged period of ischemia precondition the kidney to ischemic injury as in ischemic preconditioning. This approach aims at avoiding unintermittent exposure to ischemia for 30–45 min and minimizing tissue damage than expected through ischemic preconditioning mechanism.

The present study was conducted to test a previously examined specific method that was used to eliminate or minimize ischemic injury. The kidney was exposed to intermittent I-R cycles (8 min of clamping and 2 min of unclamping for a total of 30 min) instead of using unintermittent ischemia (30 min), and this approach relies on releasing blood flow to the kidney and thereby minimizing kidney damage. It is hypothesized that intermittent periods of ischemia, instead of unintermittent, prolonged periods of ischemia would result in less extensive tissue damage. This approach also seems to be beneficial as it allows the detection of the hemorrhagic vessels during intermittent periods of reperfusion and effective ligation of these structures during periods of ischemia. This would allow gaining more effective control over the small vessels during resection and minimizing renorrhaphy sutures following the resection, and thereby contribute to reducing ischemia in the intact kidney tissue.

NGAL and KIM-1, which significantly reflect the degree of acute kidney damage, were used as markers to compare the degree of damage between the groups.

NGAL, also known as lipocalin 2 or *lcn2*, is found to be elevated in urine and serum in the early phases of ischemic injury (2–4 h) and peaks at 24–48 h.<sup>[19,20]</sup> It is one of the first upregulated genes in acute ischemic conditions both in humans and rats. In addition, in the present study, urinary NGAL levels were significantly increased in the sham operation, intermittent ischemia, and unintermittent ischemia groups. Although serum NGAL levels were numerically increased in the sham operation, intermittent ischemia, and unintermittent ischemia groups, the differences between the groups did not reach statistical significance.

KIM-1 is a transmembrane protein, the expression of which is increased in ischemic or nephrotoxic renal tubular damage.<sup>[21,22]</sup> Urinary KIM-1 is one of the most sensitive and specific markers in differentiating ischemic kidney damage from prerenal azotemia and chronic kidney failure.<sup>[22]</sup> It becomes detectable in urine and serum within the first 24 h following ischemic injury. In the present study, urinary KIM-1 levels were significantly increased in the sham operation, intermittent ischemia, and unintermittent ischemia groups. Although serum KIM-1 levels were numerically increased in the sham operation, intermittent ischemia, and unintermittent ischemia groups, the differences between the groups did not reach statistical significance.

Serum creatinine starts to increase at 24–48 h after I-R injury in rats, the levels of which become the most

significant between days 3 and 7; serum creatinine has become the most widely used marker of kidney damage both in human and animal studies.<sup>[23]</sup> It has been used for long years in humans to demonstrate ischemic and nonischemic damage both in the short term and long term. The rats were sacrificed at 24 h to measure specific markers of kidney damage (NGAL and KIM-1), the levels of which significantly increase at 24 h. Hence, serum creatinine was evaluated at a time point that can be considered early (24 h), and this is one of the limitations of the present study. Furthermore, evaluating of creatinine levels in rats and humans with bilateral functional kidneys may result in false-positive results concerning the presence of kidney damage.<sup>[24]</sup> The glomerular filtration rate (GFR) may provide more significant results at this point, but there are obvious technical difficulties in evaluating GFR in rats.<sup>[25]</sup> Although serum creatinine levels were numerically increased in the sham operation, intermittent ischemia, and unintermittent ischemia groups, the differences between the groups were not statistically significant.

One of the other limitations of the present is that other FORs, aside from NGAL, KIM-1, and creatinine levels, were not measured to evaluate kidney damage. The present study had both infrastructural drawbacks and financial concerns, which are planned to be eliminated in human studies in the future.

One of the strengths of the present study is the surgical technique used in rats. Most of the similar studies have used a 3–4-cm midline abdominal incision to open the peritoneal cavity and expose the kidneys. Such a technique involves unnecessary interventions such as traction and suspension of bowel and other intraabdominal organs and thus produces extra surgical stress.<sup>[26]</sup> In the present study, dorsolumbar fascia was accessed, and the kidney was exposed through a left vertical flank incision using muscle-spacing dissection without cutting the muscles. The procedure was then completed by closing the skin in most of the rats.<sup>[26]</sup>

When compared to the sham operation group, the magnitude of injury being higher in the intermittent ischemia group and the presence of less extensive damage in the unintermittent ischemia group support the hypotheses of the present study. The study demonstrates that the injury is less extensive if the renal artery is declamped to allow reperfusion with a specific interval instead of clamping the artery for 30 min without interruption. With the support of these findings, the present study should be followed by human studies. In addition to the markers as mentioned earlier, evaluation of FORs and scintigraphic examination

in the long-term follow-up period (6–12 months) in human studies would be beneficial to obtain information about the functions of the operated kidney and the contralateral kidney. The I-R injury occurring during partial nephrectomy can be further reduced to the point that the present studies demonstrate. This may reduce the incidence of acute and chronic renal failure following such surgery, minimize patients' suffering, reduce the costs, and may have an economic contribution. The results that can be obtained from such studies may be extended to other organs that are exposed to ischemic injury.

## CONCLUSION

The present study evaluated the extent of kidney damage using urinary and serum markers in an experimental model of intermittent and unintermittent warm renal I-R injury. Urinary NGAL and KIM-1 levels were significantly higher in the unintermittent ischemia group than in the intermittent ischemia and sham operation groups. Urinary NGAL and KIM-1 levels were also significantly higher in the intermittent ischemia group than in the sham operation group. Serum NGAL, serum KIM-1, and serum creatinine levels were numerically higher in the sham operation, intermittent ischemia, and unintermittent ischemia groups in parallel to the levels in urine, although the differences among the groups did not reach statistical significance.

In conclusion, the rat model of intermittent I-R injury was associated with less extensive kidney damage when compared to the unintermittent I-R model.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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