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Effect of complete hilar versus only renal artery clamping on renal histomorphology following ischemia/reperfusion injury in an experimental model

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Abstract. – OBJECTIVE: To evaluate the effect of temporary complete hilar versus only renal artery clamping with different duration of warm ischemia on renal functions, and possibly identify a "safe" clamping type and duration of renal ischemia.

MATERIALS AND METHODS: Fifty male rabbits have been incorporated to study. Rabbits were subjected to ischemia/reperfusion injury by temporary vascular clamping. Reagents were randomized to 3 experimental groups (only renal artery clamping, complete hilar clamping, sham surgery) and sub-groups were determined according to different clamping times (30 and 60 minutes). Median laparotomy and left renal hilus dissection were performed to sham group. Only artery or complete hilar clamping was performed for 30 or 60 minutes by microvascular bulldog clamps to other reagents. Rabbits were sacrificed 10 days after primary surgery and left nephrectomy performed. Nephrectomy materials were evaluated for the level of nitric-oxide synthase (NOS) immunoreactivity, malondialdehyde (MDA) level and superoxide dismutase (SOD) activity and an electron microscopic examination was performed.

RESULTS: NOS immunoreactivity was correlated with the temporary clamping time. We also observed that complete hilar vascular clamping entails an increase on NOS immunoreactivity. MDA levels were similar for all experimental surgery groups ($p = 0.42$). The SOD activity was decreased among all subgroups compared with sham surgery. But the significant decrease occurred in 30 minutes only artery and 30 minutes complete hilar clamping groups in proportion to sham surgery ($p = 0.026$ and $p = 0.019$, respectively).

CONCLUSIONS: This current study suggested that only renal artery clamping under 30 minutes is more appropriate during renal surgical procedures requiring temporary vascular clamping.

Key Words:
Temporary renal vascular clamping, Ischemia/reperfusion injury, Nephron-sparing surgery, Nitric oxide synthase immunoreactivity, Superoxide dismutase activity, Malondialdehyde level.

Introduction

There has been a migration to smaller, lower-stage renal tumours by the wide use of screening techniques[1]. Nephron sparing surgery (NSS) has become an encouraging minimally invasive surgical procedure for treating these selected small renal tumours and provides recurrence-free rates similar to radical nephrectomy and prevention of renal functions[2,3]. Renal hilar clamping during NSS enables almost a bloodless surgical field to obtain accurate tumour excision, watertight closure of the collecting system, shorter operative time and minimal blood loss[4,5]. However, ischemia/reperfusion (I/R) injury is the primary reason for post-operative acute renal failure in relation to temporary renal hilar clamping during NSS[6]. The increased level of reactive oxygen species (ROS) as a result of I/R injury are neutralized by enzymatic and non-enzymatic pathways. But when an unexpected increase occurs in the amount of free radicals or when the cellular antioxidant defense systems are insufficient, ROS may induce oxidative stress in cellular components and genetic material, causing cellular dysfunction and death[7].

Nitric oxide (NO) is an inorganic free radical and a substantial mediator of renal I/R injury pathophysiology[8] and NO level in the tissue is di-

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rectly associated with hypoxia. Nitric oxide synthases (NOS) has been defined as the liable enzyme for the synthesis of NO from L-arginine and oxygen in mammalian tissues[9] and NOS inhibitors were used to treat renal warm I/R injury[10]. Malondialdehyde (MDA) is a reactive aldehyde and lipid peroxidation end-product. Superoxide dismutase (SOD) catalyses the dismutation of superoxide (O_2^-) and is an important defense system in cells exposed to oxygen. The role of this lipoxidation product and anti-oxidant enzyme following renal I/R injury were analyzed in an experimental setting and shown to be worthwhile markers for renal I/R injury[7]. In clinical practice, only renal artery clamping during NSS for a reasonable duration is supposed to be better for recovery of renal function after surgery. However, the most of clinical studies regarding renal ischemia have not decided on a time limit for pedicle clamping, yet. In this experimental study, our purpose was to analyze the effect of different renal pedicle clamping models on recovery of the renal function after surgery and determine the upper tolerable limits in warm ischemia for kidney.

Materials and Methods

Animal Preparation and Experimental design

After approval of the local Ethics Committee, a total of 50 male New Zealand rabbits, in weight of 3,000 to 3,200 g (3121 ± 79 g), were included in this study. Rabbits were housed at air-filtered, normal heat stress (20-22°C), and light-controlled (12-h light/dark cycle) room with a relative humidity of 50-52%. Diet consisted of commercial pellets and water *ad libitum*. Twenty-four hours before the procedure, food access was denied, but free access to water was continued. Anesthesia was maintained with the intramuscular injection of 70 mg/kg of ketamine and 10 mg/kg of xylazine and allowed to breathe room air spontaneously. After, a 10-min stabilization period, a midline laparotomy was performed.

Reagents were clustered in 3 experimental groups and 2 sub-groups. Group 1 (n:20), are subjected to only left renal artery clamping for 30 (group 1a, n:10) and 60 (group 1b, n:10) minutes. Group 2 (n:20) received complete left renal hilar clamping for 30 (group 2a, n:10) or 60 (group 2b, n:10) minutes. Group 3 (n:10) was sham operated, without an interference to renal vascular structures. After the ischemic period, the clamp was removed and reperfusion was achieved. Ten days after the primary operation under the same anesthesiological protocol, left nephrectomy through a midline incision was performed and the animals were allowed to exsanguinate after harvesting procedure for the renal specimens. Hemi-nephrectomy was performed throughout the hilar axis. Upper pole heminephrectomy materials were used for NOS immunoreactivity investigation and electron microscopic examination, lower poles used for MDA level and SOD activity analysis.

No pharmacological agent was used to reduce the effects of renal ischemia. For all groups, the average duration of anesthesia and surgical procedures were conducted using the same anesthetic agents.

Tissue Processing and NOS Immunohistochemistry

Tissue processing was performed as described by Oktem et al[11]. Tissue pieces were fixed in 2% paraformaldehyde (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for 48 h, at 4°C and processed for embedding in paraffin wax (Sigma-Aldrich Chemie Gmbh, Munich, Germany) using routine protocols. 5 μm-thick coronal sections were cut using a microtome (Leica MR 2145, Heerbrugg, Germany); they were, then, dewaxed and rehydrated through a graded ethanol (Sigma-Aldrich Chemie Gmbh, Munich, Germany) series using routine protocols. Sections were, then, washed with distilled water and phosphate buffered saline (PBS) (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for 10 minutes, then treated with 2% trypsin (Sigma-Aldrich Chemie Gmbh, Munich, Germany) in 50 mM Tris buffer (Sigma-Aldrich Chemie Gmbh, Munich, Germany) (pH 7.5), at 37°C, for 15 minutes. Sections were delineated with a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for 15 minutes to inhibit endogenous peroxidase activity. Then, sections were incubated with primary antibodies (Sheep Polyclonal Anti-NOS, Novus Biologicals, Littleton, CO, USA) 30 min. at 4°C in a humid chamber. Sections were then incubated with biotinylated secondary antibody (Donkey Polyclonal anti-Sheep IgG (H and L), Novus Biologicals, Littleton, CO, USA) and then with streptavidin conjugated to horseradish peroxidase (both from Zymed Histostain-plus Peroxidase-kit, 85-9043, San Francisco, CA, USA) prepared ac-

cording to manufacturer's instructions for 30 minutes each. Finally, sections were incubated with 3,3-diaminobenzidine (DAB) (from Dead-End Colorimetric TUNEL system, Promega, Madison, WI, USA, prepared according to manufacturer's instructions) for 5 minutes to reveal immunolabelling. All dilutions and thorough washes between stages were performed using PBS. Sections were counterstained with Mayer's hematoxylin (Zymed Laboratories, San Francisco, CA, USA). After washing with tap water, sections were dehydrated through a graded ethanol series, cleared in xylene and mounted with entellan (Merck, Darmstadt, Germany) Negative control samples were processed as described above except that primary antibodies were omitted and replaced with PBS alone.

Evaluation of Labelling

Immunoreactivity of NOS were assessed semi-quantitatively (Olympus BX-51, Tokyo, Japan) and Olympus C-5050 digital camera (Olympus, Bethesda, MD, USA). Two independent observers, blinded to clinical findings, separately evaluated the immunolabeling scores. The intensity of immuno-histochemical staining was graded according to the nuclear and cytoplasmic immunoreaction. Labeling intensity was graded semi-quantitatively and a histochemical score (HSCORE) was calculated using the equation HSCORE =$\sum$ Pi (i+1), where *i* = intensity of labeling with a value of 1, 2 or 3, (weak, moderate or strong, respectively) and Pi is the percentage of labeled cells for each intensity, varying from 0% to 100%[12].

Electron Microscopic Examination

Tissue processing and electron microscopic examination were performed as described by Turgut et al[13]. Tissue samples were immediately fixed in glutaraldehyde and paraformaldehyde combination pH 7.4 for 2 hours at 4°C. They were post-fixed in 1/1 ratio osmium tetroxide (OsO4) (Sigma-Aldrich Chemie Gmbh, Munich, Germany) + Cacodylate added buffer (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for 2 hours. Following dehydration in a series of graded alcohols placed twice in propylene oxide (Sigma-Aldrich Chemie Gmbh, Munich, Germany), and then subsequently embedded in Epon-812 (SPIChem., West Chester, PA, USA). Following polymerization, transverse thin sections were cut on a microtome (Reichert, Austria Nr. 313864) and stained with 1% toluidine blue (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for light microscopy. Transverse thin sections were cut with glass knives and doubly stained with 0.5% uranyl acetate (Sigma-Aldrich Chemie Gmbh, Munich, Germany) and 0.2% lead citrate (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for ultrastructural examination. Sections were placed on Gilder Grids G 200 3.05 mm diameter copper grids (Sigma-Aldrich Chemie Gmbh, Munich, Germany). The sections were examined with the transmission electron microscope (Zeiss EM 9 Zeiss, Jena, Germany) and the electron micrographs were taken for histological evaluation.

Biochemical Analysis

Lower-pole hemi-nephrectomy tissue samples were immediately transferred to the laboratory under cold chain. Gerota fascia and perirenal fat tissue were removed. Renal tissue samples were homogenized for determination MDA levels and superoxide dismutase activity.

Determination of MDA Levels in Renal Tissue Homogenates by Using TBARS Mmethod

MDA levels measured spectrophotometrically at 530-540 nm by using thiobarbituric acid method. According to the method, tissue samples homogenized in 1000 □L RIPA buffer (Sigma-Aldrich Chemie Gmbh, Munich, Germany) (with PMSF) (v:v, 1:8) by using disposable homogenization tubes (Xiril Dispomix, Xiril AG., Hombrechtikon, Switzerland) and homogenized samples were centrifuged at 1600 g, + 4°C for 10 minutes. Supernatants were used for analysis and results were given as nanomol/g protein.

Determination of Total Protein Level and Superoxide Dismutase Activity

Renal tissue samples were homogenized in PBS (Sigma-Aldrich Chemie Gmbh, Munich, Germany) (v: v; 1:8) by using Xiril Dispomix homogenizator (Xiril Dispomix, Xiril AG. Hombrechtikon, Switzerland). Homogenized samples were centrifuged at +4°C, 400 g for 10 minutes. Supernatants were removed and 2 ml 1 × cell extraction buffer (10 × SOD buffer, 20% Triton-X, 200 nM phenylmethylsulphonyl fluoride (PMSF, Sigma-Aldrich Chemie Gmbh, Munich, Germany) in distilled water, were added to the samples. Samples were incubated on ice for 30 minutes. Then samples were centrifuged at 10000 for 10 minutes at +4°C. Supernatants were used for SOD activity and total protein measure-

ment. Total protein levels were measured in a microplate reader (Thermo Multiscan microplate reader, Thermo Fisher Scientific Inc, Franklin, MA, USA) by using BCA protein assay reagent (Pierce, Rockford, IL, USA) at 490 nm. SOD activity was measured by using Assay Design kit according to manufacturer's instructions (SOD activity kit, Cat # 900-157, Assay Designs Inc., Ann Arbor, MI, USA). Briefly, the kit is based on consecutive measurements WST formazan color crystals at 450 nm with one-minute intervals for 10 minutes. Slope were measured by using absorbance changes in minutes. SOD activity was measured by using below formula and SOD standard curve: % inhibition = [(1 × SOD control buffer slope – sample slope) × 100]/1 × SOD control buffer slope.

Statistical Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences Version 16.0 software program (SPSS 16.0 for Windows, SPPS Inc., Chicago, IL, USA). HSCORE was calculated for NOS immunoreactivity and One-way ANOVA was used for statistical analyses. Binary analyses were evaluated by Tukey test. MDA levels and SOD activity were treated as continuous variables and they are presented as mean ± standard deviation (SD). Then, data were compared among and within groups using One-way ANOVA with control and two-way ANOVA. Differences were considered to be significant at p values less than 0.05.

Results

MDA Level and SOD Activity

Mean MDA levels and SOD activity are shown in Table I. Mean MDA levels did not reveal a significant difference between groups (p = 0.42). Mean SOD activity revealed a marginal difference among all groups regardless of from clamping type/period (p=0.07). However, statistical analysis with two-way ANOVA, revealed a significant difference between group 1a, group 2a and sham operated group (p=0.026 and p=0.019, respectively) when one type of variable (clamping time/period) was kept constant. Group 1b and group 2b did not show a significant difference with sham operated group (p = 0.27 and p = 0.29, respectively) in terms of SOD activity.

Increasing Warm Ischemia Duration and Complete Hilar Clamping Encourage Nos Immunoreactivity

Group 3 showed very weak NOS immunoreactivity in glomerules, proximal and distal tubules, henle loop and collecting ducts. The number of immunopositive cells were less than 10% (Figure 1a). Group 1a revealed weak NOS immunoreactivity and the number of immunopositive cells were less than 10%. The signal intensity of proximal tubules were weak but distal tubules showed moderate immunoreactivity (Figure 1b). There was a significant difference with all groups (p < 0.05) except sham operated group (p = 0.514) (Figure 2). Group 1b showed increased NOS immunoreactivity and the number of immunopositive cells were less than 50%. Glomerules, proximal and distal tubules represented moderate immunoreactivity (Figure 1c). There was a significant difference with all groups (p < 0.05) except group 2a (p = 0.162) (Figure 2). Group 2a showed strong NOS immunoreactivity and the number of immunopositive cells were less than 50%. The intensity of glomerules were strong. Proximal tubules showed less immunoreactivity than distal tubules (Figure 1d). There was a significant difference with all groups (p < 0.05) except group 1b (p

Table I. Mean MDA levels and SOD activity.

Groups	SOD (Unit/g protein)	MDA (µM/g protein)
Only artery clamping		
30 minutes (Group 1a)	33.2 ± 7.5*	17.8 ± 3.6
60 minutes (Group 1b)	42.3 ± 19.7	18.9 ± 2.7
Complete hilar clamping		
30 minutes (Group 2a)	31.4 ± 9.9**	16.1 ± 3.3
60 minutes (Group 2b)	42.8 ± 17.2	19 ± 2.8
Sham operated (Group 3)	52.9 ± 20	19.1 ± 4

*p = 0.026, Between group 1a and group 3, in terms of SOD activity **p = 0.019, Between group 2a and group 3, in terms of SOD activity.

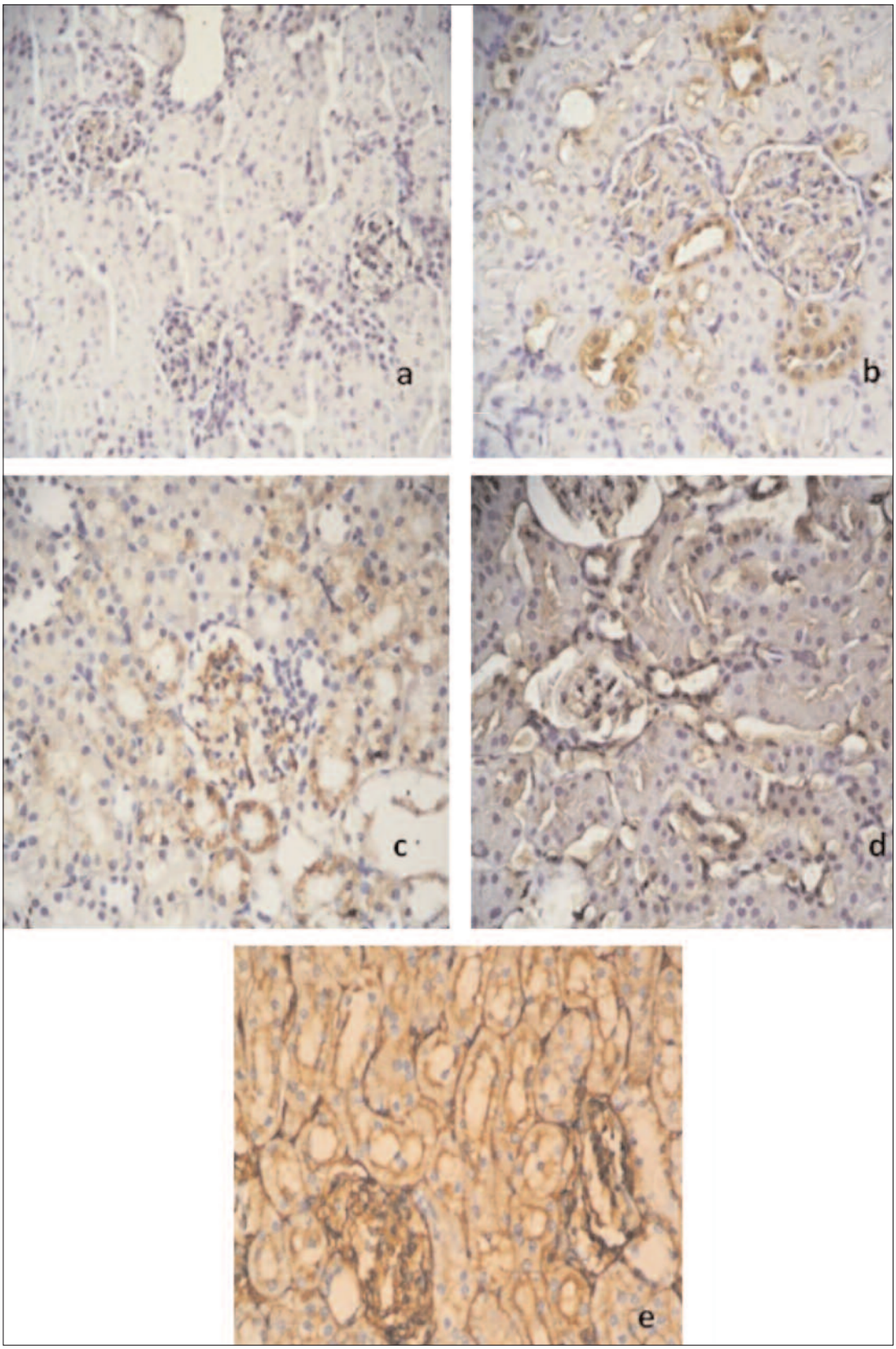

Figure 1. NOS immunoreactivity in experimental groups: ***a,*** Sham-operated group, ***b,*** Only artery clamping for 30 min. ***c,*** Only artery clamping for 60 min. ***d,*** Complete hilar clamping for 30 min. ***e,*** Complete hilar clamping for 60 min.

= 0.162) (Figure 2). Group 2b revealed the strongest NOS immunoreactivity and the number of immunopositive cells were greater than 50%. The signal intensity revealed a prominent NOS immunoreactivity in glomerules, proximal and distal tubules, henle loop and collector ducts (Figure 1e). There was a significant difference between group 2b and all groups ($p < 0.05$) (Figure 2).

Electron Microscopic Examination

Borders of cells were clear and cytoplasm volume was normal in the control group. Gyratus proximal tubule cells were observed with a well-developed basal compartment and uniform morphological appearance in the proximal segment of the nephron. Pedicels of podocytes were settled on capillary basal lamina with normal morphological appearance. Capillaries were surrounded by a thin endothelium. Deterioration in nucleus borders, increased cytoplasmic vacuolization and cellular degeneration observed in group 1a. The degenerations became more apparent in group 1b. Cytoplasmic vacuolization, degenerative images in mitochondria cristae and a decrease in the number of mitochondria were seen in group 2a, as same as group 1b. The formation of chromosomal condensation and the demilune shape of chromosomes suggested that some of the cells entered the early phase of apoptosis. The most degenerative changes were observed in group 2b. More sections were seen with impaired showing the basal membrane integrity. An increase of intracellular vacuolization was most apparent in group 2b. Nuclear notches and cytoplasmic retractions suggested the late stage of apoptosis. Also, cell volumes increased significantly in this group, cytoplasm became more slim and fragmented in some sections (Figure 3).

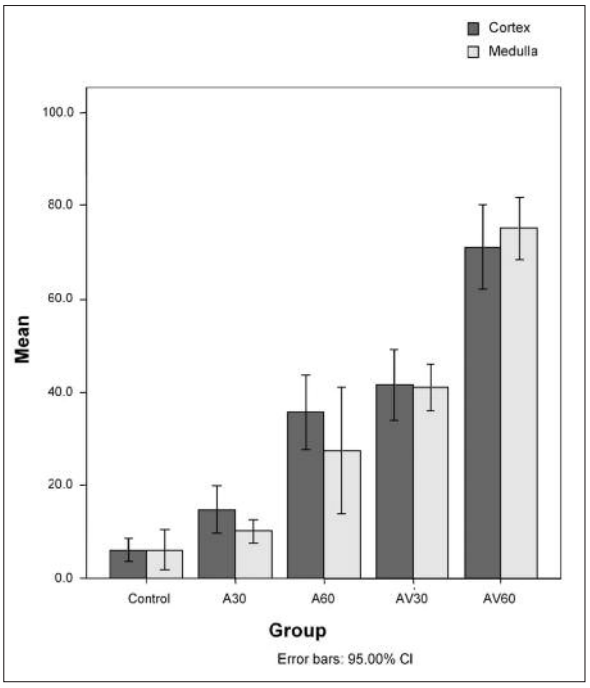

Figure 2. Evaluation of NOS immunoreactivity by HSCORE. (A30 & A60: only artery clamping for 30 and 60 min; AV30 & AV60: Complete hilar clamping for 30 and 60 min). $p < 0.05$ between control group, A60, AV30 and AV 60 groups; AV30 and AV60; AV60 and other groups. $p = 0.514$ between control group and A30. $p = 0.162$ between A60 and AV30 group.

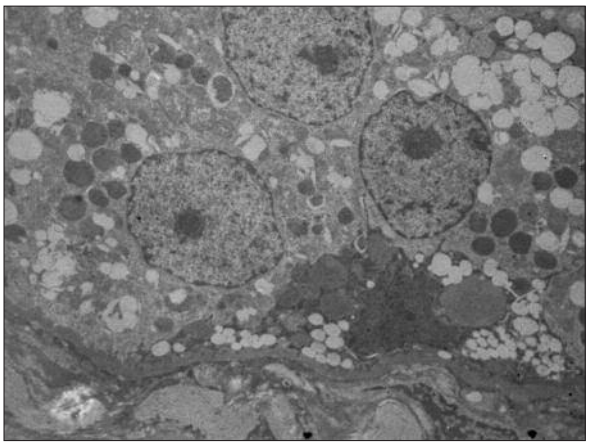

Figure 3. Electron microscopic section of complete hilar clamping for 60 min (group 2b).

Discussion

In our knowledge, there are no sufficient data to identify the upper tolerable limits in warm ischemia including different renal vascular clamping type and duration during NSS. This study reports an experimental model of warm renal ischemia, in respect to temporary vascular clamping. Our findings suggested only artery clamping for less than 30 minutes during NSS is a reasonable approach to preserve the remaining renal unit. Renal recovery after I/R injury was assessed by the biochemical measurements (SOD activity and MDA level), electron microscopic exam and semiquantitave immunohistochemistry methods for NOS.

Some animal studies[14,15] have shown that artery only clamping preserves resistance of kidney to warm ischemia better than complete hilar clamping, but histological and functional effects could not be fully demonstrated. In the review of literature on the subject, as it can be seen from most of the studies about open or laparoscopic partial nephrectomy, usually focused on the duration of ischemia, but did not evaluate the effects of different models of temporary vascular clamping[5,15-17]. Although there is no consensus about the duration of warm ischemia due to temporary renal vascular clamping, exposure below 30 minutes is considered to be better for recovery of renal function after surgery[5,17].

Functional effect of renal hilar clamping was shown by measurement of serum creatinine values in an experimental study, but histopathological effects on renal tissue were not been demonstrated[15]. In addition, all groups were exposed to a single duration (120 minutes) of warm ischemia in this study. Role of ROS, anti-oxidant enzymes and lipid peroxidation products such as MDA on I/R injury has been shown by experimental studies[9]. I/R injury causes an increase in the level of ROS and lipid peroxidation products, and reduction of anti-oxidant defense systems[9]. The effect of some agents such as L-carnitine, DHEA-S, L-arginine, kefir and *Gingko biloba* EGb761 extract on the recovery of renal function after I/R injury were examined[6,7,19-22]. In our study, we did not use any agent which has known to have a renoprotective or renotoxic effect. We only examined the pure effect of different vascular clamping types and duration on renal histology and functions.

SOD is an anti-oxidant enzyme which has an important role on removal of ROS. We investigated the association between SOD activity and temporary vascular clamping time. The SOD activity was decreased among all subgroups compared with sham surgery. This data is consistent with the literature[6,7]. The significant decrease occurred in group 1a and group 1b in proportion to sham surgery ($p = 0.026$ and $p = 0.019$, respectively). This finding may indicate prolonged warm ischemia period may allow new enzyme synthesis.

The level of oxidative damage following I/R injury was assessed by MDA levels, a lipid peroxidation end-product. MDA levels were similar for all experimental groups and did not reveal a significant difference between groups ($p = 0.42$). MDA level content was assessed in two different studies and was found significantly higher in the I/R injury group than in the sham-operated group[8,9]. Reperfusion period of time was limited to 24-72 h after renal vascular clamping in these studies. The reperfusion periods are shorter in comparison to our study. This result may point relatively prolonged reperfusion period allows removal of lipid peroxidation products such as MDA. The data obtained from our study indicates that in the presence of relatively prolonged reperfusion period, MDA may not be a safe indicator for I/R injury.

The role of NO in renal I/R injury is still ambiguous. However, it has been shown that the increase in NOS activity leads to the decrease of I/R injury[6,19]. In addition, L-arginine, the NO precursor, was provided as an agent that attenuates the development of long-term complications of I/R injury[19]. Moreover, it has been demonstrated that protective effects of ozone on acute renal failure regarding I/R injury were closely concerned with the renal NO production after rise of the endothelial NOS and inducible NOS expression[6]. We assessed the NOS immunoreactivity for detecting the effect of I/R injury of kidney in a warm ischemia model and determined that increasing clamping time is correlated with NOS activity. The increase in NOS immunoreactivity was most clear in group 2b. Furthermore, complete hilar clamping was found to cause an increase in NOS immunoreactivity independently from clamping duration. We also examined the I/R injury induced ultrastructural changes and identified that NOS activity is correlated with cell damage. These findings suggested that evaluation of NOS may be a safe marker even after a relatively long process of I/R injury. However, further experimental studies are needed in this regard.

In addition to all these arguments, one can speculate that the effect of 60 minutes of warm renal ischemia is of little clinical interest, because various clinical series report 30 minutes or less ischemic period. Furthermore, some of current series reported "zero ischemia" during partial nephrectomy procedure[21,22]. Gill et al[21] reported zero ischemia during laparoscopic/robotic partial nephrectomy with the combination of hypotensive anesthesia technique. They have claimed that controlled hypotension induced by some anesthetic agents may overcome the complications related to hemorrhage induced (uncontrolled) hypotension and hemodynamic responses are widely different between these conditions.

However, pharmacologically induced hypotension requires extensive monitorisation and an expert anesthesiologist in this regard. Despite the usage of all these invasive monitorisation techniques is not always possible to provide a 'controlled' systemic blood pressure. Isoflurane, one of the agents used for pharmacologically induced hypotension has been shown to decrease blood flow through pre-portal area and brain-stem with some changes in the renal sympathetic nerve activity[23]. Additionally, superselective artery clamping to ensure zero ischemia during robotic partial nephrectomy also has been shown to increase the mean operative time significantly ($p < 0.001$) compared with main artery clamping which may concretize the complications of the hypotension[24]. Furthermore, numerous clinical and basic research studies[25-27] have reported or investigated temporary renal ischemic periods up to 60 minutes during NSS. There is no doubt that the duration of the renal ischemia should be restricted as short as possible. However, in daily surgical practice temporary renal ischemic period may be longer than expected, as can be observed in the literature[26,27]. Therefore, in this study we have also analysed the effect of 'relatively' long warm ischemia period (60 minutes) on I/R injury and recovery of renal function.

In a interesting study, Formiga et al[28], have investigated the effect of artery only or totally hilar clamping on renal functions by 99m-Technetium-mercaptoacetyltriglycine scintigraphy. The warm ischemia duration was 80 minutes. As a distinction from other studies, they have performed intermittent totally hilar clamping and discovered that only artery and intermittan totally hilar clamping preserves renal function rather than totally hilar clamping. These findings are consistent with the present work.

Conclusions

Our findings suggested only artery clamping for less than 30 minutes during NSS is even better for recovery of kidney after I/R injury. The findings obtained from this study might encourage further research on this topic, especially focusing on clinical outcomes.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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