A novel approach to off-clamp partial nephrectomy demonstrates significant improvements in renal injury in an experimental porcine model

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Cite as: *Can Urol Assoc J* 2017;11(10):E390-5. http://dx.doi.org/10.5489/cuaj.4305
Published online October 10, 2017

Abstract

Introduction: We sought to design a partial nephrectomy (PN) with contralateral total nephrectomy porcine model and assess the underlying mechanisms of ischemia reperfusion injury (IRI) after PN using a novel, clinically approved resection device.

Methods: Domestic male pigs (n=9) underwent left lower pole PN, allocated to either standard (Group 1) or no ischemia PN (Group 2), followed by contralateral nephrectomy. Biochemical studies were performed at baseline, Day 2, and Day 7; after sacrifice, kidneys were processed for histological analysis. Apoptotic markers were measured by Western blot analyses. Urinary biomarkers were measured to assess acute kidney injury.

Results: At Day 2 following PN, there was a significant rise in serum creatinine in Group 1 compared to Group 2 (355 vs. 136 mmol/L; p=0.008). Intra-renal tissue oxygen saturation after PN was inversely correlated with postoperative creatinine (r_s -0.75; p=0.012) and the grade of acute tubular necrosis (r_s -0.70; p=0.036). We observed a rise in expression of pro-apoptotic markers and pro-inflammatory markers in Group 1 following PN compared to Group 2. Histological analysis revealed higher grade of apoptosis in Group 1.

Conclusions: IRI associated with standard PN has a deleterious impact on acute renal function, markers of tissue injury, and histological parameters, compared to off-clamp PN using the ALTRUS device. We identified several intraoperative and postoperative markers that may be used as predictors for functional and histological injury following PN.

Introduction

The management of renal tumours has evolved over the last years. More lesions are found incidentally after imaging studies performed for other medical reasons. Partial nephrectomy (PN) is considered the standard of treatment for small renal masses (SRM) with no particular surgical approach defined.[1,2] Several surgical and technological innovations have been developed to increase the chances of performing an ideal PN,[3] where oncological outcomes and renal function preservation are crucial elements. The functional benefit of PN relies on the quantity and quality of nephrons in the remaining kidney. Warm ischemia time is a surgical modifiable factor that affects postoperative renal function. This has been proven to be particularly important in patients with pre-existing chronic kidney disease.[4]

Ischemia-reperfusion injury (IRI) leads to acute tubular necrosis (ATN), which may cause subsequent renal function deterioration; however, the acute biochemical, intracellular, and histological mechanisms behind these clinical findings are not fully understood. The utility of tools, such as real-time capillary perfusion,[5] as well as various biomarkers, including KIM-1, NGAL and circulating H_2S, have been reported to be predictive of tissue injury;[6-9] however, their utility in a PN model has not previously been described. For this reason, we designed a porcine model to evaluate the difference in overall renal function, animal recovery, markers of cellular injury, and histological changes following PN using either standard renal pedicle clamping or off-clamp technique using a novel resection tool that is currently in clinical use. In order to avoid the possible confounding role of the contralateral renal unit, we used an extreme PN model whereby the contralateral kidney was also removed at the time of PN in order to maximize the burden on the remaining kidney. We hypothesized that animals undergoing off-clamp PN would have better immediate renal function, with reduced levels of pro-inflammatory and pro-apoptotic markers of cellular injury and a minimized degree of histopathological damage compared to the standard PN control group.

Methods

The study protocol was approved by the Animal User Subcommittee of Western University (AUP 2014-004).

Thirteen male domestic pigs were assigned into two different groups. Group 1 underwent left lower pole 1/3 PN with vascular pedicle clamping for 45 minutes; Group 2 had off-clamp left lower pole 1/3 PN. All animals had a contralateral nephrectomy performed immediately after the reconstruction of the left renal parenchyma. Recovery was assessed daily; pigs had free access to food and water and all of them had the same IV fluids protocol on postoperative Day 1 and 2. Sacrifice was carried out on postoperative Day 7 to evaluate the integrity of the urinary tract and harvest the remaining of the left renal parenchyma for further analysis. Biochemical, acute kidney injury (AKI) markers, and hydrogen sulphide (H_2S) levels in whole blood and urine were evaluated before and immediately after surgery, and at postoperative Day 2 and 7, before sacrifice.

Surgical technique

After prophylactic antibiotic (cefazolin 1g IV), general anesthesia with isoflurane was administered. A jugular vein catheter was placed for intra- and postoperative IV fluids, medications, and collection of samples. Buprenorphine (0.02 mg/kg) and a fentanyl patch were administrated for pain management. A midline incision was used and the left retroperitoneum was accessed by extraperitoneal approach. Left kidney was mobilized and upper ureter was identified. Hilar dissection allowed vessel loop placement in renal vein and artery. Renal parenchyma was measured and a 4 cm mark measured from the tip of the lower pole was defined as the site of transection in the lower pole; this approximated our 1/3 PN in both groups. Volume of the resected specimen was measured by water displacement. In the standard PN group, the renal hilum was controlled using bulldog forceps and resection was carried out with metzenbaum scissors. The hilum was clamped for 45 minute, as per previous porcine PN models,[10,11] to recreate a common clinical approach. In the off-clamp PN group, resection was completed using the novel ALTRUS Thermal Tissue device (10 mm, ConMed, Canada), which has the advantage of sealing and resecting parenchyma simultaneously. After parenchymal transection, the exposed collecting system was oversewn with 4-0 PDS figure of eight stitches and a lower pole reconstruction was carried out using a two-layer 3-0 PDS running suture and a Surgicel (Ethicon, Sommerville, NJ, U.S.) bolster to close the renal defect. The rationale to create this left PN with contralateral total nephrectomy porcine model was to ensure that all post-PN renal function was attributable to the remaining renal unit, thus allowing the evaluation of IRI effects on the renal parenchyma subjected to ischemia exclusively. Although this model is likely more severe than most clinical scenarios, its use was particularly chosen to better reflect the influence on IRI, in the setting of PN, on the remaining nephrons.

Biochemical evaluation included serum and urinary parameters such as creatinine, urea, electrolytes, and urine protein to creatinine ratio (UPC). Urinary and whole blood H_2S levels were measured using a microsulfide ion electrode (Lazar Research Laboratories, Los Angeles, CA, U.S.) and blood:urine ratio calculated.[9] Urinary levels of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) were evaluated in urine by ELISA and the concentration was normalized dividing the values by the urine creatinine concentration.[12] Intraoperative Doppler ultrasound aided in the evaluation of peak systolic velocity (PSV) and resistive index (RI) of the renal artery, before and 10 minutes after renal reconstruction or reperfusion in case of standard PN. Near-infrared spectroscopy (NIRS) using InSpectra StO2 (Hutchinson, MN, U.S.) allowed the evaluation of oxygen tissue concentration in renal parenchyma before and 15 minutes after reperfusion in Group 1 PN or at the same time point following off-clamp resection in Group 2. After sacrifice, the left kidney specimens were bivalved and processed for histological evaluation and protein analysis by Western blot. An independent pathologist reviewed the specimen in hematoxylin and eosin (H&E) and created a scale (0–100%) to determine the grade of ATN. Histological sections were also stained with terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) to assess the degree of apoptosis. Sections were imaged at five random areas using a Nikon Eclipse 90i light microscope at 10x magnification. The number of positively stained cells per field of view were quantified using Image J software (National Institute of Health).

Western blot analysis of the kidney tissue samples for apoptotic markers

Total proteins were isolated from the kidney tissues using a protein and RNA isolation system kit (Ambion, Life Technologies, U.S.) according to the manufacturer's instructions. Afterwards, proteins were visualized by enhanced chemiluminescence kit (Milipore, U.S.) on Fluorchem gel documentation system (Protein Simple, U.S.).

Statistical analysis

We evaluated biochemical, clinical, hemodynamic, inflammatory, and histological characteristics according to the type of surgery. A comparison between biochemical characteristics of both groups was performed using non-parametric tests. A Students t-test and Fisher's exact test were used to evaluate dichotomous variables. Correlations were analyzed using Spearman's rank correlation coefficient. All p values were from two-tailed tests and $p<0.05$ was considered statistically significant; data was presented in medians and interquartile range (IQR) or range. Statistical analysis was performed using IBM SPSS Statistics for Windows, Version

20.0 (Armonk, NY, IBM Corp). Quantification of band density in Western blot analysis was determined using the Image J software package.

Results

A total of 13 pigs (n=8, standard PN Group 1; n=5, off-clamp PN Group 2) were included in the experiment; however, only nine pigs completed the postoperative evaluation until the day of sacrifice (n=4, standard PN; n=5, off-clamp PN) and were included in the final analysis. We found that following this extreme model of PN, three of the standard PN animals died even before the first blood draw and only four survived, whereas all of the animals who underwent off-clamp PN completed the study. This highlights the physiological impact that a standard PN may have, especially in patients with impaired renal function or solitary kidneys. Baseline characteristics were similar among the two groups.

The amount of parenchymal resection was similar between both groups (17 cc); however, estimated blood loss (EBL) was greater in Group 2 (18 vs. 75 ml; p=0.008). As expected, the immediate postoperative parenchymal microcapillary oxygen saturation was significantly lower in the standard PN group. Animals subjected to standard PN had a more torpid course, which was reflected in the postoperative biochemical evaluation, where serum creatinine was significantly higher compared to Group 2, although median levels of serum creatinine eventually returned toward baseline near the end of the experiment in both groups. No significant changes in serum creatinine were found in Group 2 during the study period (Fig. 1). Table 1 shows the preoperative and postoperative characteristics for both groups. Regardless of the type of surgery, the renal RI and tissue microcapillary oxygen tissue tension measured before sacrifice were similar. Urinary H_2S levels were different at Day 7 between both groups, but this difference was not statistically significant (p=0.14). Blood H_2S levels markedly increased after IRI in Group 1 and returned to baseline levels at Day 7, while H_2S levels remained stable throughout the experiment in in the off-clamp PN group.

Analysis of urinary biomarkers of acute kidney injury revealed a pronounced elevation of NGAL and KIM-1 in Group 1 on postoperative Day 2 and 7; however, only the elevation in NGAL persisted at the end of the experiment (p<0.05), whereas KIM-1 levels returned back to control levels. Ratio of blood:urine H_2S levels mirrored that of KIM-1 (p<0.05). Interestingly, none of acute markers of renal tubular injury were affected by off-clamp PN, highlighting its importance in mitigating renal injury (Fig. 2).

At the time of sacrifice, post-mortem evaluation did not reveal any surgical complications, including the presence of any urinoma, hematoma, or hydronephrosis. Histological analysis revealed similar levels of ATN in both groups evaluated by H&E staining; however, TUNEL staining showed a higher level of apoptosis in Group 1 compared to Group 2, which did not reach significance (p=0.053). The expression of pro-apoptotic markers Bid and MAPK were significantly elevated in the standard PN group compared to the off-clamp group (p<0.05, Fig. 1), and although other markers, including Bax and caspase-3, were also found to trend towards a difference, they were not statistically significant. We performed a correlation analysis between intraopera-

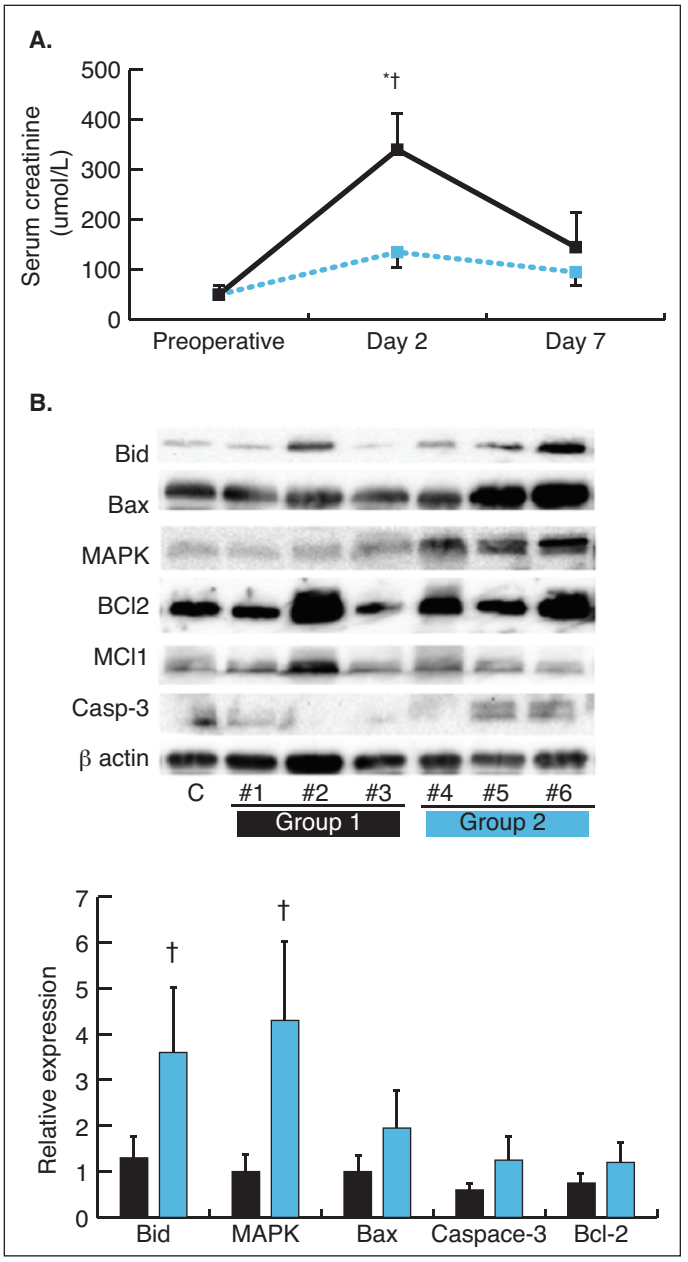

Fig. 1. (A) The effect of standard (black) vs. off-clamp (blue dotted) partial nephrectomy on serum creatinine. ***(B)*** Representative expression of apoptotic markers by Western blot analysis. There was a significant increase in Bid and MAPK following standard partial nephrectomy. *p<0.05 compared to preoperative levels; †p<0.05 compared to off-clamp partial nephrectomy.

Table 1. Comparison between baseline and postoperative characteristics in animals undergoing either off-clamp or standard partial nephrectomy (PN)

	Standard PN	Off-clamp PN	p
Preoperative characteristics			
Weight (kg)	32	32	1
Urea (mmol/L)	3.15	2.5	0.55
Creatinine (umol/L)	54	60	0.42
Blood H_2S (nmol/L)	1.52	1.74	0.69
Urine creatinine (mmol/L)	7.05	8	0.69
Urine protein, U g/L	0.41	0.44	0.55
Urine H_2S (nmol/L)	0.74	1.26	1
Renal parenchymal StO_2% before PN	94	96	0.22
Renal resistive index	0.7	0.68	1
Intraoperative and post-reconstruction assessment			
Volume of parenchymal resection (cc)	17	17	1
Estimated blood loss (ml)	18	75	**0.008**
Renal parenchymal StO_2% after PN	80	93	**0.008**
Median change in StO_2% before and after PN	-17	-4	**0.008**
Serum creatinine (umol/L)			
Day 2	335	136	**0.008**
Day 7	133	112	0.73
Blood H_2S (nmol/L)			
Day 2	40.7	1.8	**0.04**
Day 7	12.6	1.9	0.14
Urine H_2S (nmol/L)			
Day 2	2.7	1.3	0.21
Day 7	4.1	1.1	0.14

tive and postoperative and histological variables. Intrarenal tissue oxygen saturation after PN inversely correlated with Day 2 serum creatinine (r_s -0.75; p=0.012) and the grade of ATN (r_s -0.70; p=0.036). Additionally, blood H_2S levels obtained from Group 2 pigs were inversely correlated with the grade of apoptosis (r_s -0.787; p=0.020), and urine protein concentration ratio at Day 7 correlated with ATN (r_s 0.866; p=0.003), suggesting these acute markers could be used as a predictor of renal parenchymal tissue injury.

Discussion

In our extreme model of PN and IRI, we observed a significant increase in renal injury as measured by intrarenal microcapillary perfusion, serum creatinine, and several biochemical markers of tissue and tubular injury, including blood:urine H_2S ratio, KIM-1, and NGAL levels. For the first time, we were able to demonstrate that intraoperative parameters, such as renal microcapillary tissue oxygen tension, correlated with postoperative biochemical and histological parameters of IRI in a porcine model.

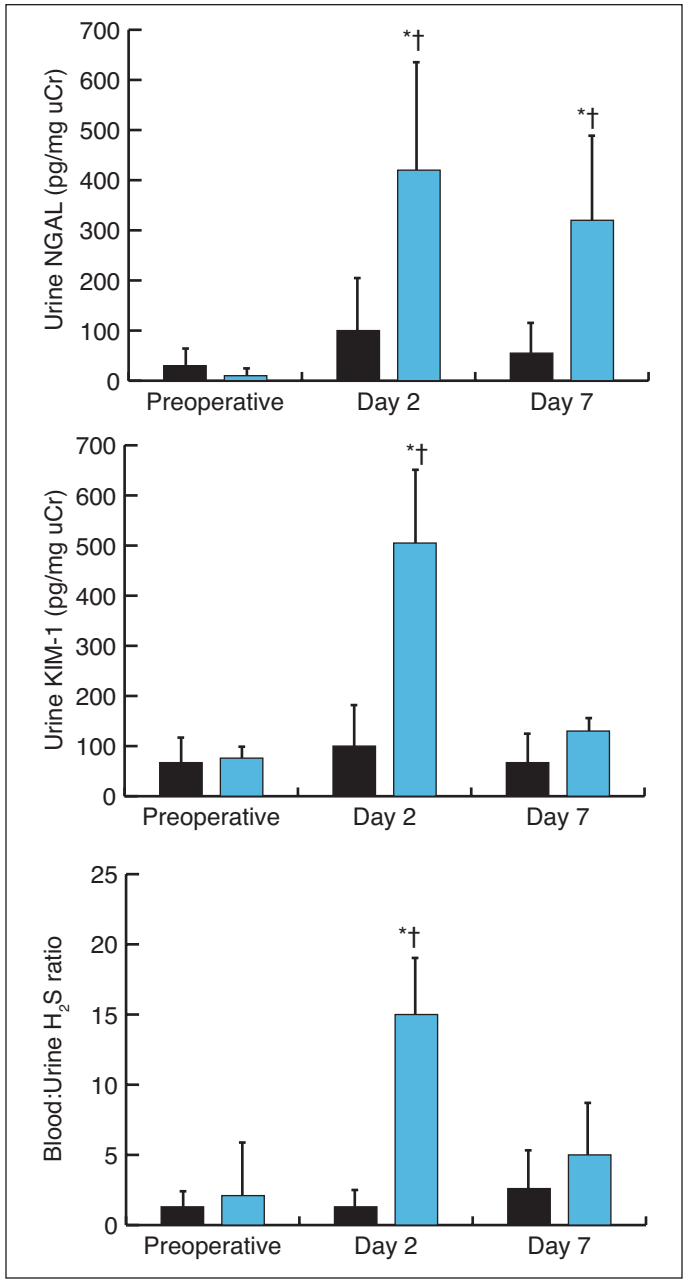

Fig. 2. The effect of off-clamp (black) and standard (blue) partial nephrectomy on urinary biomarkers of tissue injury NGAL, KIM-1, and blood:urine H2S ratio over time. *p<0.05 compared to preoperative levels; †p<0.05 compared to off-clamp partial nephrectomy.

Our data demonstrated a significant increment in serum creatinine after standard PN, although by the end of the study, serum creatinine levels had returned toward baseline. These findings suggest renal tissue recovery after the IRI, which is not unexpected at seven days post-injury. In fact, the observed rise in urine KIM-1 and blood H_2S suggests that these mediators may have been involved in tissue regeneration and repair on Day 2 post-PN, enabling a gradual return to normal function in the standard PN arm.[13-15] H_2S blood levels

inversely correlated with the grade of apoptosis assessed through TUNEL stain. The histological analysis demonstrated similar levels of ATN, but the grade of apoptosis was slightly higher in the group subjected to IRI, a finding supported by the elevated expression of pro-apoptotic markers on Western blot analyses.

Diverse clinical and animal studies have evaluated the usefulness of acute kidney injury biomarkers, such as NGAL and KIM-1, in the detection and prognosis of renal injury after surgery; however, there is no clear consensus as to what clinical consequence these elevated levels translate to.[16-18] Increased expression of these biomarkers was observed in our experiments, and KIM-1 was significantly elevated after IRI compared to off-clamp PN. This biomarker has been proven to be more sensitive than serum creatinine for AKI diagnosis and it may be a better method of evaluating renal function in the setting of IRI. In fact, several studies have suggested a direct relationship between KIM-1 expression and the grade of histological injury, specifically in patients with chronic kidney disease.[7,19,20] Additional experimental studies are needed to ascertain whether KIM-1 could potentially help to determine which patients benefit most from PN compared to radical nephrectomy.[21] The use of blood:urine H_2S ratio has not been used to predict renal tissue injury previously; however, it clearly demonstrated a mirroring of KIM-1 activity, suggesting that it could one day be used as an inexpensive and real-time assay of renal injury without having to perform expensive and difficult ELISA assays; further testing will be required to evaluate this in a larger series.

Interestingly, despite the differences in biochemical and histological markers of renal tissue injury, especially on postoperative Day 2, the overall renal function was similar after seven days. This finding demonstrates the recovery capacity of healthy renal tissue, emphasizing the importance of the quality of nephrons to determine the new baseline renal function.[21] When extrapolated to the clinical setting, where the majority of renal cell carcinoma patients are older and have comorbidities predisposing them to chronic renal insufficiency or baseline chronic kidney disease, our findings regarding the importance of off-clamp PN become even more relevant. We are the first group to use NIRS in renal tissue parenchyma during PN to evaluate microcapillary perfusion changes associated with IRI, demonstrating an inverse correlation between the tissue oxygenation immediately after PN and the grade of ATN and serum creatinine despite similar warm ischemia time in the standard PN group. This assessment could potentially reflect a more accurate stratification of the IRI than warm ischemia time and deserves further clinical evaluation as a surrogate to predict long-term effects of IRI. Indeed, in using this extreme model of PN, we were able to demonstrate the detrimental effects of renal pedicle clamping, as five of the nine animals in the standard partial nephrectomy group died in the first two days following surgery, suggesting that the renal injury was too great for recovery. This becomes very important when considering a standard approach to PN in patients with renal insufficiency.

We chose to evaluate the ALTRUS resection device for the purposes of these experiments, as the design of the jaws of the instrument enables a simultaneous and equal pressure closure of both tips in a "pant-press" fashion rather than the usual "crocodile jaws" closure seen with other energy devices. This design feature enabled us to generate a controlled pressure over the renal parenchyma with minimal tearing and shearing, while maintaining excellent hemostasis, thus making it ideal for the purposes of the off-clamp PN. This device has previously been reported to be used in a variety of cases in the literature, including appendectomy.[22] Its utility in the clinical setting of off-clamp PN remains to be seen, but this data strongly highlights its feasibility and potential benefits.

This study was not without its limitations. We envisioned this experiment as a pilot study with a small number of animals per arm to minimize animal usage, which may have affected the results, although we used non-parametric tests to allow for a better statistical evaluation of our findings. In addition, although the porcine model is a common PN model, it is also known to be less vascularized than the human kidney.[23]

Conclusion

Off-clamp PN using the ALTRUS device was successfully performed in this pilot study, which is the first of its kind to exemplify its utility in the setting of renal surgery. A comprehensive evaluation of the mechanisms behind the tissue injury observed in the standard PN arm allowed us to determine several predictors of histological injury. Our hypothesis was corroborated by finding a better immediate renal function (Day 2), and reduced levels of AKI biomarkers and pro-apoptotic cellular markers of tissue injury in the off-clamp group, compared to standard PN group. We also show, for the first time, potential prognostic and therapeutic targets of tissue injury incurred during PN that require further assessment.

Competing interests: Dr. Sener has received an investigator-initiated research grant from PerfuseMED and is the cofounder of Clearwater Clinical Ltd. The remaining authors report no competing personal or financial interests.

Acknowledgement: We thank CONMED Canada for their support in supplying the ALTRUS devices, as well as for the support of the investigator-initiated study.

This paper has been peer-reviewed.

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