

Does Aliskiren Protect the Kidney Following Ischemia Reperfusion Injury?

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Summary

The effect of blocking the first and rate-limiting step in renin-angiotensin cascade on the renal function in ischemia reperfusion injury has not been previously investigated. We investigated the effect of aliskiren, the first approved direct oral renin inhibitor, on the alterations in renal functional parameters in this condition. Wistar rats underwent left renal ischemia for 40 min. Group-1 received normal saline whereas Group-2 received aliskiren (30 mg/kg/day) by gavage for 6 days commencing one day before IRI. The hemodynamic and tubular functions and gene expression of neutrophil gelatinase-associated lipocalin (NGAL) and plasminogen activating inhibitor (PAI-1) in the right and left kidneys were measured five days following the IRI. Comparing Group-1 and Group-2, the left renal blood flow was significantly higher in Group-2 (1.28 ± 0.36 vs. 0.39 ± 0.05 , $P=0.007$). Left kidney glomerular filtration rate was also higher in Group-2 but did not reach statistical significance (0.18 ± 0.05 vs. 0.10 ± 0.02 , $P=0.07$). The left renal FE_{Na} was significantly lower in Group-2 (29.9 ± 6.4 vs. 49.7 ± 7.8 , $P=0.03$). Aliskiren also caused a significant decrease in the gene expression of both NGAL and PAI-1 in the left ischemic kidney. In conclusions, the administration of aliskiren before and after IRI appears to have ameliorated the IRI effect on the total renal artery blood flow, and fractional excretion of sodium and gene expression of both NGAL and PAI-1 indicating a renoprotective effects in IRI.

Key words

Ischemia-reperfusion injury • Aliskiren • Renal function

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Introduction

Renal ischemia-reperfusion injury (IRI) is an invariable consequence of several conditions including renal transplantation (Weight *et al.* 1996). It has been well-established that IRI causes renal functional alterations which might ultimately result in renal impairment (Axelsen and Cartwright 1979, Brady *et al.* 1996). Several substances have been involved in this process such as the elements of the renin-angiotensin system (RAS) (Kontogiannis and Burns 1998, Hammad *et al.* 2001, Habibey *et al.* 2008, Fouad *et al.* 2010). In this regard, both angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) were shown to ameliorate the ischemia-reperfusion induced renal injury (Habibey *et al.* 2008, Molinas *et al.* 2009, Fouad *et al.* 2010).

The RAS consists of several peptides which interact in a cascade fashion to produce angiotensin II. Renin is the enzyme which is catalyzing the metabolism of angiotensinogen to angiotensin I, which is subsequently converted by the action of ACE to the active hormone angiotensin II. Therefore, the effect of the RAS can be blocked by interrupting this cascade at different levels by agents such as ACE inhibitors and ARBs. RAS blockade can also be achieved by renin inhibitors. Renin inhibition is expected, at least theoretically, to be more effective than the ACE inhibitors or the ARBs in blocking the function of RAS. This is probably due to the fact that renin controls the first and rate-limiting step in RAS. In addition, RAS blockade by ACE inhibitors or by ARBs has been shown to cause a compensatory increase in renin release which might decrease the potential effects of these agents

(Johnston *et al.* 1979, Jones *et al.* 2007). This theoretical effectiveness of renin inhibitors was supported by the findings that the renal vasodilator response to the renin inhibitor enalkiren exceeded the effect of the ACE inhibitor captopril (Cordero *et al.* 1991, Fisher *et al.* 1994).

Historically, renin inhibitors have not been clinically successful because of the lack of potency or poor bioavailability. Aliskiren is the first oral direct renin inhibitor which has been approved for the treatment of hypertension by the US Food and Drug Administration. The effect of aliskiren on kidney function in different renal conditions has been studied (Whaley-Connell *et al.* 2010, Wu *et al.* 2010, Choi *et al.* 2011). In IRI, aliskiren has been recently shown to reduce renal fibrosis in a mice model of chronic ischemic kidney injury (Sun *et al.* 2012). However, the effect of aliskiren on the alterations in renal functional parameters following IRI has not been investigated yet. Thus, the aim of this study was to investigate this effect in a rat model of warm acute IRI.

Materials and Methods

Studies were performed in male Wistar rats weighing 211–261 g at the time of IRI. Rats were housed in standard cages and kept in a 12-h light-dark cycle at 20 °C. They were fed a standard rat chow and had free access to water. Animals were fasted for 12 h before the experimental procedures but had water *ad libitum*. The experimental protocol was approved by the local animal research ethics committee.

Ischemia-reperfusion injury

The following procedures were carried out under aseptic conditions. Animals were anesthetized with ketamine hydrochloride (70 mg/kg, intraperitoneally, Pantex Holland B. V., Holland) and Pentobarbital Sodium (20 mg/kg, intraperitoneally, Sigma Life Science, St Louis, USA). With minimal dissection, the left renal artery was exposed *via* a flank incision and was occluded using microvascular non-traumatic bulldog clamp. Following a warm ischemia of 40 min, the microvascular clamp was removed to allow reperfusion. At the end, the wound was closed in layers.

Aliskiren/ Vehicle administration

Aliskiren (Novartis Pharma Stein AG, Stein, Basle, Switzerland) was dissolved in 0.5 ml of normal saline and administered by gavage immediately after preparation as single daily dose of 30 mg/kg. Control

animals received only 0.5 ml of normal saline. In the two groups, treatment was commenced 24 h before ischemia and continued daily for 5 days thereafter until the time of the terminal experiment.

Experimental groups

Animals were randomized into two groups:

1. Group-1 (n=10): Rats which underwent renal ischemia and received only normal saline.
2. Group-2 (n=9): Rats which underwent renal ischemia and received aliskiren.

Surgical procedure in the terminal experiment

All rats underwent terminal experiments five days following IRI. The details of surgical procedure were similar to what has been previously described (Hammad *et al.* 2012). In brief, after induction of anesthesia and tracheal cannulation, a femoral artery and vein were cannulated. Both kidneys were then exposed through a midline abdominal incision and the upper ureters were cannulated for the collection of urine into pre-weighed micro-capped tubes. The urine volume was determined gravimetrically.

On completion of surgery, rats were infused with a solution composed of fluorescein isothiocyanate-inulin (FITC-inulin, Sigma-Aldrich, St Louis, USA) (2.5 mg/ml) and para-aminohippuric acid (PAH, Sigma-Aldrich, St Louis, USA) (0.2 % w/v) in 0.9 % saline at a rate of 50 ml/min. A priming dose of 2 ml of the same solution was infused over 2 min. Animals were allowed 2 h to equilibrate before being subjected to the experimental protocol.

Experimental protocol and assays

The experimental protocol which consisted of two 30-min clearance periods was similar to what has been described previously (Hammad *et al.* 2012). The urine and plasma samples were assayed for sodium level and the inulin and PAH contents were determined. The values of Glomerular filtration rate (GFR) and renal blood flow (RBF), urine volume (UV), urinary sodium ($U_{Na}V$) and fractional excretion of sodium (FE_{Na}) were calculated as the average of the two clearance periods and were corrected for kidney weight.

Gene expression analysis

At the end of the experimental protocol, the kidneys were removed. Part of each kidney was excised, immediately snap-frozen in liquid nitrogen and stored at

-80 °C for a later measurement of the relative gene expression of neutrophil gelatinase-associated lipocalin (NGAL) and plasminogen activating inhibitor (PAI-1).

Total RNA was extracted using TRI Reagent® Solution (Life Technologies Corporation, NY, USA) according to the manufacturer protocol. Quality and quantity of the extracted RNA was estimated using NanoDrop instrument (Thermo Fisher Scientific Inc., DE, USA). First-strand cDNAs were prepared in duplicates from 1 µg of the extracted RNA using MuLV reverse transcriptase (Applied Biosystems, CA, USA) and served as template for relative gene expression analysis which was also performed in duplicates on ABI-7500 Real Time PCR System (Applied Biosystems, CA, USA) using

TaqMan chemistry. The reaction mixture consisted of 50 ng cDNA, PCR Gold buffer, 0.375 U AmpliTaq Gold DNA polymerase (Applied Biosystems, CA, USA), MgCl₂ 6.0 mM, Rox dye 50 nM, DMSO 0.4 %, Triton X-100 0.04 %, Nonidet P-40 0.04 %, Tween-20 0.04 %, forward and reverse primers and FAM-BHQ-1 labelled probe (Biosearch Technologies, Inc., CA, USA). Sequences and concentrations of primers and fluorogenic probes are listed in Table-1. Primers and probes were designed using the online RealTimeDesign™ software (Biosearch Technologies, Inc., CA, USA) in a way that at least one of the primers was spanning an exon-intron junction within their respective gene.

Table 1: Forward and reverse primers and fluorogenic probe sequences used for real time quantitative PCR analysis.

Gene		Sequence	Concentration (nM)
<i>RPLP0</i>	Forwad Primer	CAGAGGTGCTGGACATCACAG	300
	Reverse Primer	CACATTGCGGACACCCTCTAG	900
	FAM-BHQ-1 Probe	CAGGCCCTGCACACTCGCTTC	150
<i>PAI-1</i>	Forward Primer	GGCACAATCCAACAGAGACAA	600
	Reverse Primer	GGCTTCTCATCCCCTCTCAAG	300
	FAM-BHQ-1 Probe	CCTCTTCATGGGCCAGCTGATGG	150
<i>Lcn2 (NGAL)</i>	Forward Primer	CTGTTCCCACCGACCAATGC	900
	Reverse Primer	CCACTGCACATCCCAGTCA	900
	FAM-BHQ-1 Probe	TGACAACACTGAACAGACGGTGAGCG	150

Raw fluorescence data generated by the ABI-7500 Real-Time PCR System was subjected to analysis using the Real-time PCR Miner analysis software (Zhao and Fernald 2005). Calculated reaction efficiencies and CTs were then used to calculate the relative gene expression using the delta-delta CT methodology. Results for the gene expression were normalized with 60S acidic ribosomal protein P0 gene expression as a house keeping gene.

The results were expressed as the relative gene expression of both NGAL and PAI-1 in the left ischemic kidney compared to the right control kidney in the respective group.

Histological studies

The right and left kidneys from the two groups

(total: 38 kidneys) were fixed in 10 % buffered normal saline, dehydrated in increasing concentrations of ethanol, cleared in xylene and embedded in paraffin. Five µm sections were stained with hematoxylin and eosin. Histological examination and scoring were performed blindly by a pathologist (second author). A numerical score was used to define the degree of tubular cell necrosis as described previously (Mohan *et al.* 2006) (Score 0: no necrosis; Score 1: few focal necrotic spots; Score 2: tubular necrosis in approximately half of the field; Score 3, tubular necrosis in approximately two thirds of the field; Score 4: approximately the entire field was necrotic). All kidney sections were examined and a numerical score was reported for each kidney. An average score was then calculated for each group.

Statistical analysis

Statistical analysis was performed using SPSS V16.0. Results were expressed as means \pm SEM. One-way factorial ANOVA was used for comparison of variables between the two groups and between the control and ischemic kidneys within each group. P value of less than 0.05 was considered statistically significant.

Results

The mean arterial blood pressure (122 ± 3 vs. 121 ± 3 mm Hg, $P=0.8$) and heart rate (455 ± 10 vs. 451 ± 6 bpm, $P=0.7$) in Group-1 and Group-2 were similar.

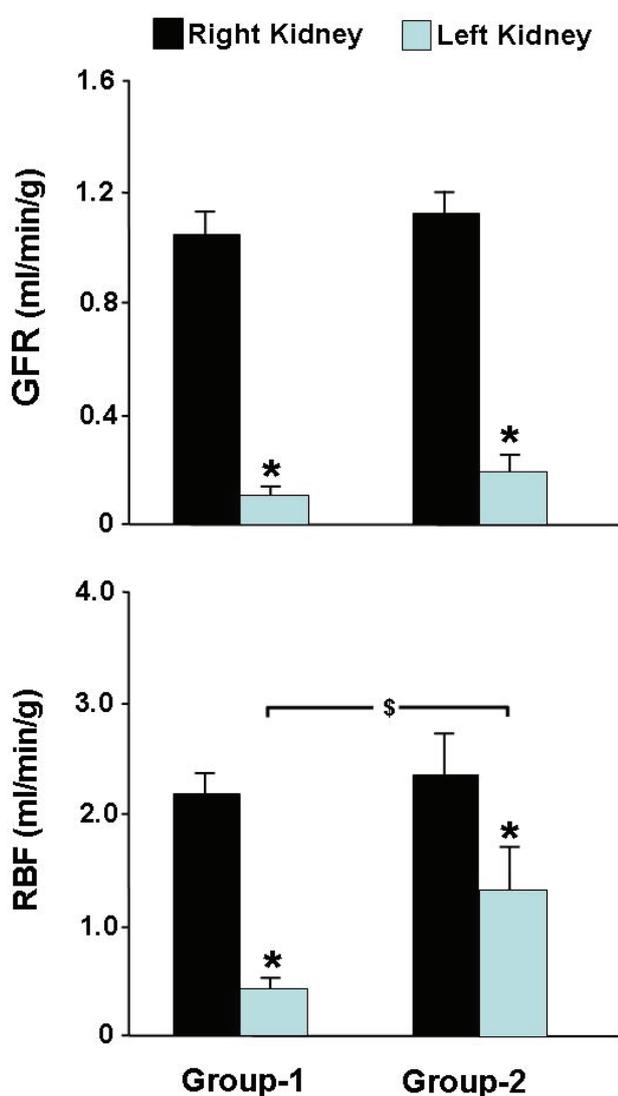


Fig. 1. The glomerular filtration rate (GFR) and renal blood flow (RBF) in the right and left kidneys in Group-1 and Group-2 following 40 min of left renal ischemia. Values represent mean \pm SEM. * indicates statistical significance between the right and left kidney within the same group whereas \$ indicates statistical significance between the left kidneys in both groups.

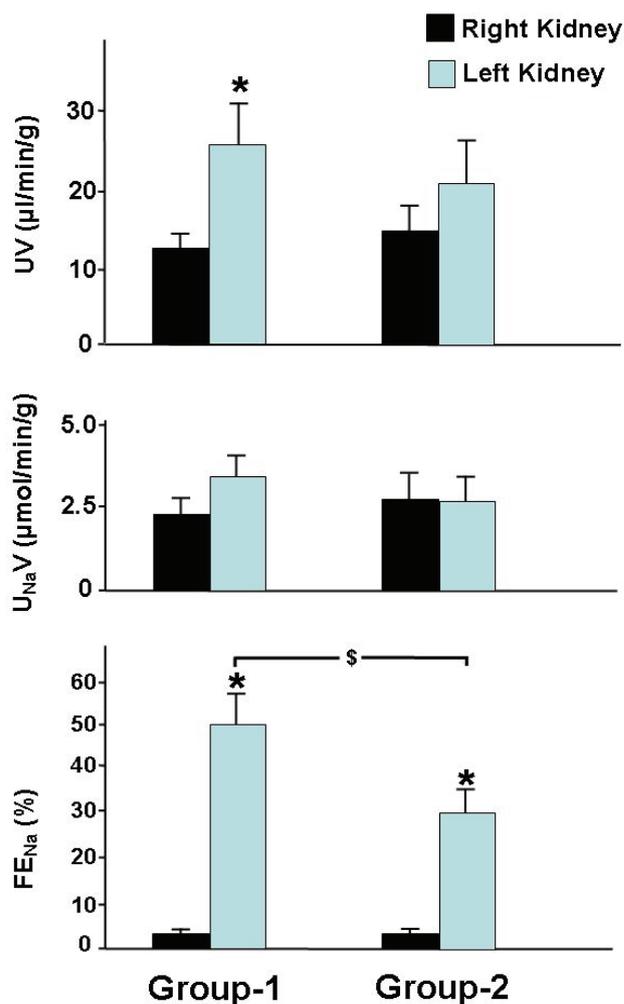


Fig. 2. The tubular functional parameters including urine volume (UV), urinary sodium ($U_{Na}V$) and fractional excretion of sodium (FE_{Na}) in both kidneys in Group-1 and Group-2 following left renal ischemia. Values represent mean \pm SEM. * indicates statistical significance between the right and left kidney within the same group whereas \$ indicates statistical significance between the left kidneys in both groups.

Glomerular and tubular functions

In Group-1 which had left renal ischemia but did not receive aliskiren, left RBF, five days following the IRI, was 17 % of the right RBF (0.39 ± 0.05 vs. 2.24 ± 0.27 ml/min/g, $P < 0.0001$). Similarly, the left kidney GFR was 10 % that of the right non-ischemic kidney (0.10 ± 0.02 vs. 1.04 ± 0.11 ml/min/g, $P < 0.0001$) (Fig. 1). With the decrease in both RBF and GFR, the UV (26.7 ± 4.9 vs. 12.3 ± 1.8 μ l/min/g, $P = 0.006$) and FE_{Na} (49.7 ± 7.8 % vs. 2.9 ± 0.4 %, $P < 0.0001$) in the left kidney were significantly higher than the right kidney (Fig. 2). $U_{Na}V$ in the left kidneys was also higher than the right one but did not reach statistical significance (3.5 ± 0.7 vs. 2.3 ± 0.5 μ mol/min/g, $P = 0.07$).

In Group-2 which received aliskiren, the left

RBF was approximately half of the right RBF (1.28 ± 0.36 vs. 2.39 ± 0.45 ml/min/g, $P=0.03$) and the left renal GFR was 16% of the right GFR (0.18 ± 0.05 vs. 1.13 ± 0.09 ml/min/g, $P<0.0001$) (Fig. 1). As shown in Figure-2, the UV and $U_{Na}V$ of the left kidney were similar to those from the right kidney (21.1 ± 5.7 vs. 15.2 ± 3.3 μ l/min/g, $P=0.2$ and 2.6 ± 0.7 vs. 2.8 ± 0.8 μ mol/min/g, $P=0.4$, respectively) but the left renal FE_{Na} was significantly higher than the right kidney ($29.9 \pm 6.4\%$ vs. $3.1 \pm 0.9\%$ ($P=0.001$)).

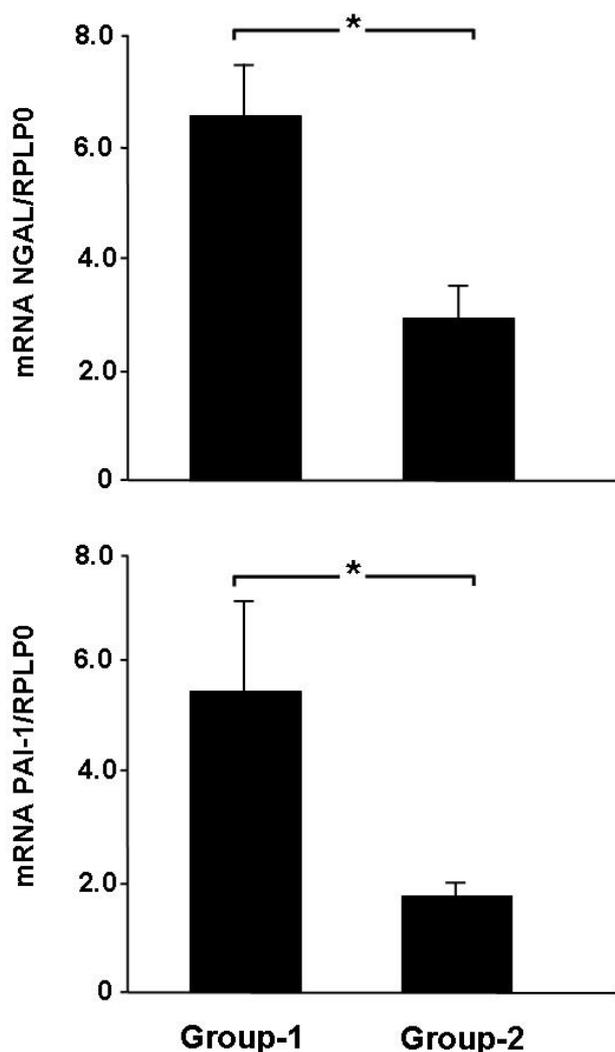


Fig. 3. The expression of NGAL and PAI-1 genes in Group-1 and Group-2 measured by real time PCR. Results were expressed as the fold increase in the gene expression in the left ischemic kidneys compared to the right non-ischemic kidney in each group. * indicates statistical significance between both groups.

When Group-2 was compared to Group-1, the left RBF was significantly higher in Group-2 (1.28 ± 0.36 vs. 0.39 ± 0.05 ml/min/g, $P=0.007$). The left GFR was also higher in Group-2 but did not reach statistical

significance (0.18 ± 0.05 vs. 0.10 ± 0.02 ml/min/g, $P=0.07$) (Fig. 1). As shown in Figure-2, left renal UV and $U_{Na}V$ were similar in both groups ($P>0.05$ for both variables). However, left renal FE_{Na} was significantly lower in Group-2 which received aliskiren ($29.9 \pm 6.4\%$ vs. $49.7 \pm 7.8\%$, $P=0.03$) (Fig. 2). All variables in the right kidneys in both groups were similar ($P>0.05$ for all variables).

Gene expression analysis results

As demonstrated in Figure 3, there was 6.5 ± 0.9 fold increase in the expression of NGAL in the left ischemic kidney compared to the right non-ischemic kidney in Group-1 whereas this increase was only 2.9 ± 0.6 fold in Group-2 ($P=0.02$). Similarly, aliskiren resulted in a significantly lower expression of PAI-1 in the ischemic kidney in Group-2 compared to Group-1 (1.8 ± 0.2 vs. 5.5 ± 1.6 , $P=0.04$).

Histological studies

The right non-ischemic kidneys in the two groups had normal architecture (score 0) (Fig. 4). As demonstrated in Figure 3, examination of the left ischemic kidneys in Group-2 has shown a less severe degree of acute tubular necrosis compared to Group-1 despite the fact that when comparing the arithmetic mean histological score of the left ischemic kidneys in the two groups (2.7 ± 0.36 vs. 3.3 ± 0.23), this showed a trend which did not reach statistical significance ($P=0.09$). This improvement was demonstrated by the fact that only five animals from group-2 had a score ≥ 3 compared to eight animals in Group-1. In addition, none of the animals from Group-1 had a score of 1 whereas there were two animals from Group-2 which had a score of 1. The number of animals with various scores in both groups were as follows: Group-1: score 4: $n=5$, score 3: $n=3$, score 2: $n=2$, score 1: $n=0$, score 0: $n=0$) and Group-2: score 4: $n=3$, score 3: $n=2$, score 2: $n=2$, score 1: $n=2$, score 0: $n=0$).

Discussion

In the current study, we have demonstrated for the first time that the administration of aliskiren prior to and following warm renal IRI has resulted in a significant improvement in some of the hemodynamic and tubular renal functional parameters and attenuated the rise in gene expression of some markers of acute kidney injury.

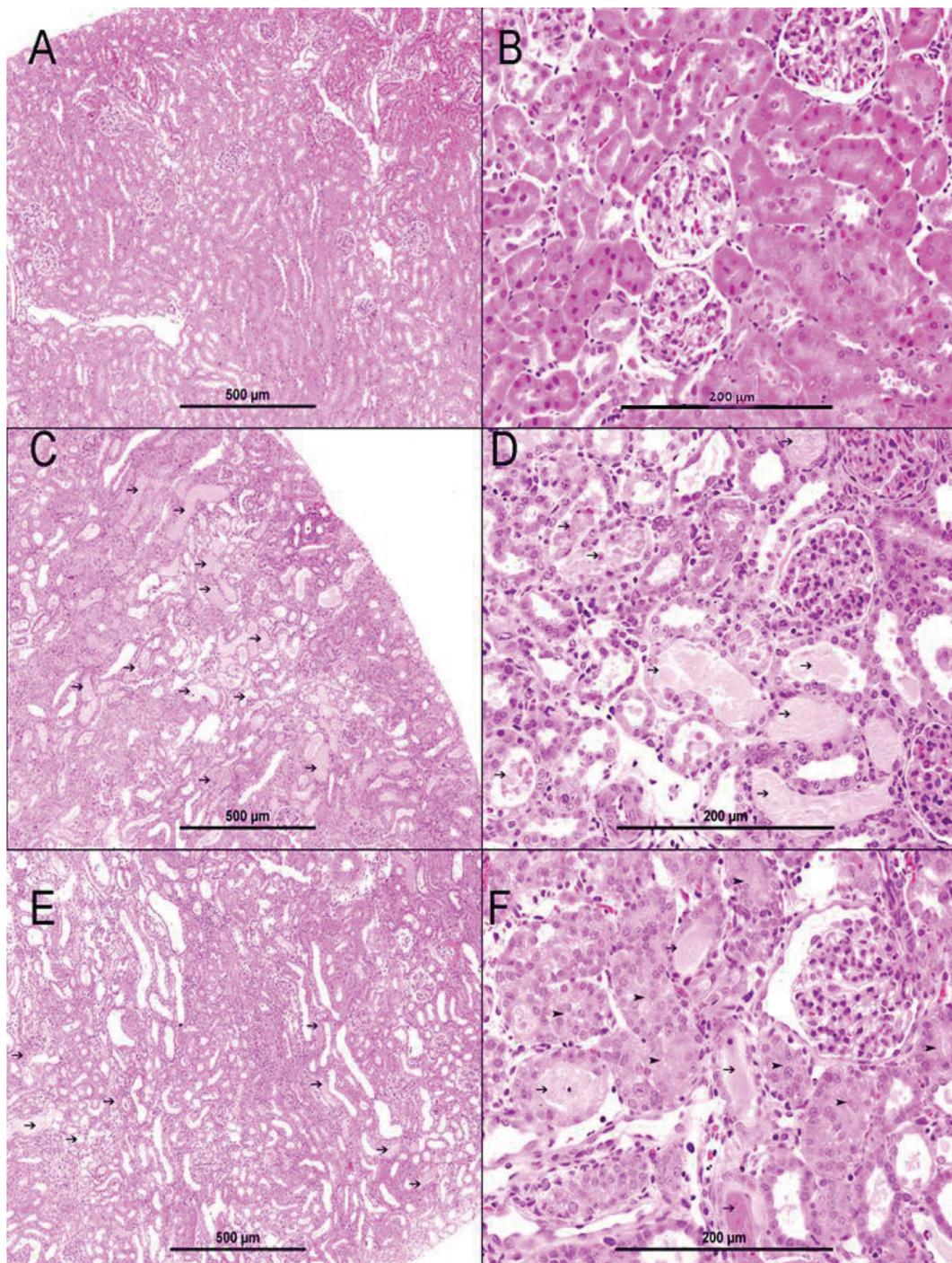


Fig. 4. Light microscopic features of the rats kidney sections (hematoxylin-eosin). **A** and **B**: right kidneys showing normal glomeruli and tubules. **C** and **D**: left kidneys from group 1 which underwent IRI without aliskiren showing acute tubular necrosis (thin arrows) involving more than 2/3 of renal parenchyma. **E**: left kidneys from group 2 which underwent IRI and received aliskiren showing improvement of acute tubular necrosis (thin arrows) with involvement half of renal parenchyma. **F**: left kidneys from group 2 also showing improvement of acute tubular necrosis (thin arrows) with presence of recovered normal renal tubules (arrow head) in some areas.

Renin-angiotensin system (RAS) plays a central role in the development of ischemia-reperfusion induced renal dysfunction. Several studies have shown that RAS blockade by ACE inhibitors or ARBs results in an amelioration of the ischemia-induced renal injury

(Habibey *et al.* 2008, Molinas *et al.* 2009, Fouad *et al.* 2010). Given that renin catalyses the first and rate-limiting step of RAS, direct renin inhibitors were shown to be effective in ameliorating the effects of RAS system in some renal pathophysiological conditions (Whaley-

Connell *et al.* 2010, Wu *et al.* 2010, Choi *et al.* 2011). In the current study, we have shown that blocking the RAS at its first step using aliskiren results in an attenuation in the ischemia-induced renal injury. Collectively, the data from the current study and from studies which investigated ACE inhibitors and ARBs indicate that blocking RAS at any level would result in an improvement of the ischemia-induced renal functional alterations. However, further studies are required to compare the degree of effectiveness of aliskiren, ACE inhibitors and ARBs in blocking the RAS and IRI-associated renal alterations.

In the current study, aliskiren improved both the RBF and FE_{Na} in the post-ischemic kidney. There was also mild improvement in the GFR although it did not reach statistical significance. The exact reason is difficult to ascertain but it could be due to the possible selective effect of angiotensin II on efferent glomerular arterioles. In this regards, the renin inhibitor aliskiren results in a decrease in the production of angiotensin II. Although angiotensin II causes constriction of both afferent and efferent glomerular arterioles, there is some evidence to suggest that it has a preferential effect on the efferent arterioles which results in the maintaining of GFR especially in pathological conditions (Hall *et al.* 1977a,b, Edwards 1983). Therefore, decreasing the level of angiotensin II by aliskiren might have resulted in eliminating this effect of selective efferent arteriolar constriction on glomerular filtration and hence a less effect on GFR with aliskiren. However, further studies are required to clarify this point.

In addition to its effect on the hemodynamic renal parameters, the current study indicated that aliskiren significantly reduced the rise in FE_{Na} which is caused by the IRI, and hence increased the ability of the kidney to concentrate urine. In this regard, several studies have previously shown that ischemia-reperfusion injury results in tubular damage, at least in the short-term, as indicated by the deterioration of several parameters such as the FE_{Na} which reflects the ability of the renal tubules to reabsorb the filtered sodium and hence the concentration ability of the kidney (Hammad *et al.* 2001, Kiew *et al.* 2004, Kirkby *et al.* 2007, Hammad *et al.* 2012). In the current study, it is unlikely that the improvement in the FE_{Na} was due the decline in the production of angiotensin-II as a result of aliskiren administration since angiotensin II is known for its antidiuresis and antinaturesis properties (Hammad *et al.* 2000). The exact cause for this improvement in the deterioration of the

FE_{Na} is difficult to ascertain but could be due the improvement in the degree of tubular necrosis as a result of the improvement in the RBF which was induced by aliskiren administration.

In this model of IRI, measurement of other parameters of tubular damage such as proteinuria would have added to the results. However, in this unilateral renal ischemic model, urine collection in the terminal experiment was performed for a relatively short period using ureteric catheterization, a technique which could have resulted in some degree of trauma to the ureteric wall and would have falsely increased the amount of protein in the collected urine.

In parallel to the improvement in the hemodynamic and tubular functions, there was also attenuation in the rise of both NGAL and PAI-1 in the ischemic kidney in the treated group at the gene expression level. NGAL is one of the acute kidney injury markers and has been shown to increase in IRI. There is an evidence to suggest that it is an important marker in determining the severity and outcome of the injury (Parikh *et al.* 2010, Mishra *et al.* 2003). PAI-1 is a fibrogenic factor which has also been implicated in the ischemia-reperfusion induced kidney injury (Favreau *et al.* 2010). The attenuation of both markers at indicates a protective effect of aliskiren at the gene expression level and more studies are required to investigate the effects of aliskiren on the long-term IRI-induced renal dysfunctions.

In the current study, kidney functions were measured five days following IRI. Early, after the IRI especially in the first 48 h, the GFR would have been minimal after similar periods of ischemia (Sabbatini *et al.* 2010, Rubinstein *et al.* 2009). Therefore, it might have been difficult to show any significant effect of aliskiren especially in view of the fact that it would have been administered for only a limited period. Certainly, administering aliskiren for five days following the injury would have given the drug reasonably enough time to exert its action. In the longer-term, despite the recovery of some renal functional parameters such as serum creatinine (Pechman *et al.* 2009), there is an evidence to suggest that some other renal functional parameters and responses to certain stimuli, might be still altered (Pechman *et al.* 2009). Further studies are required to investigate the long-term effect of aliskiren on kidney functions.

In the current study, we used a single daily dose of aliskiren. This does not only mimic the clinical setup

but also is in agreement with animal studies when the drug was also given as a single daily dose and has shown significant effect in different pathological conditions (Singh *et al.* 2008, Wu *et al.* 2010, Choi *et al.* 2011, Rusai *et al.* 2011). However, the use of aliskiren in renin inhibition in rats might have affected the drug bioavailability. Aliskiren has been shown to have a higher specificity for human and mouse rennin compared to rats (Wood *et al.* 1987). Ideally, determining a good bioavailability of aliskiren requires measuring plasma rennin activity or angiotensin II levels which was lacking in this study. Nevertheless, the significant effect of aliskiren on the attenuation of the ischemia-induced changes in RBF, FE_{Na} and gene expression of NGAL and PAI-1 indicates that bioavailability was not a very major issue in this study. These findings were generally in agreement with other studies which investigated aliskiren in rats and showed significant effects using similar or even lower doses of aliskiren (Singh *et al.* 2008, Wu *et al.* 2010, Tain *et al.* 2011).

The RBF in the current study is lower than the values obtained in our recent work (Hammad *et al.* 2012). This might have been due to the fact that a similar rate of para-aminohippuric (PAH) infusion was used in both studies, although the rats used in this study were smaller than those used in our previous work. This might have

resulted in some degree of volume expansion in the current study so that the level of PAH in the filtered load could have exceeded the PAH excretion ability by the renal tubules and hence an apparently lower RBF (Tanner and Isenberg 1970, MacDougall and Wiegmann 1988). Higher FE_{Na} in both non-ischemic and post-ischemic kidney might support this explanation. Nevertheless, the fact that the same protocol and infusion rate were used in both treated and untreated groups, both of which had an ischemic and non-ischemic kidneys, render it unlikely that this could have affected the overall results.

In conclusion, the administration of aliskiren before and after ischemia-reperfusion injury appears to have a significant protective effect on the total renal blood flow, fractional excretion of sodium and gene expression of both NGAL and PAI-1 indicating a renoprotective effect of this drug in renal ischemia-reperfusion injury.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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