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Original Article

The effect of thymoquinone on the renal functions following ischemia-reperfusion injury in the rat

Fayez T Hammad, Loay Lubbad

Department of Surgery, College of Medicine & Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates

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Abstract: Introduction: The aim of this study was to investigate the effect of thymoquinone, an antioxidant phytochemical compound found in the plant Nigella sativa, on the alterations in renal functional parameters following warm renal ischemia-reperfusion injury (IRI) in the rat. Methods: Wistar rats underwent left renal ischemia for 35 minutes. Group-TQ (n=15) received thymoquinone 10 mg/kg/day (dissolved in a vehicle (corn oil) orally by gavage starting 4 days prior to IRI and continued 6 days thereafter when the hemodynamic and tubular renal functions of the right and left kidneys were measured using clearance techniques. Group-Vx (n=15) underwent similar protocol but received only the vehicle. Results: IRI affected all hemodynamic and tubular parameters in the affected kidney. Thymoquinone attenuated the IRI-related alteration in renal functions so when the left ischemic kidney in Group-TQ and Group-Vx were compared, the left RBF and GFR were significantly higher in Group-TQ (2.02±0.39 vs. 1.27±0.21, P=0.04 and 0.33±0.08 vs. 0.18±0.03, P=0.03, respectively). Thymoquinone also improved left renal FE_{Na} (1.59±0.28 vs. 2.40±0.35, P=0.04). In addition, it decreased the gene expressions of KIM-1, NGAL, TNF-α, TGF-β1 and PAI-1 (143±20 vs. 358±49, 16±3 vs. 34±6, (1.1±0.2 vs. 2.8±0.4, 1.6±0.1 vs. 2.8±0.1, and 2.4±0.3 vs. 5.8±1.0, P<0.05 for all). Conclusion: Thymoquinone ameliorated the IRI effect on the hemodynamic and tubular renal functional parameters as well as the expression of some kidney injury markers and pro-inflammatory and pro-fibrotic cytokines indicating a renoprotective effect of this agent on the IRI-induced renal dysfunction with potential clinical implications.

Keywords: Thymoquinone, ischemia-reperfusion injury, renal functions

Introduction

It has been well-established that renal ischemia-reperfusion injury (IRI) causes renal functional alterations that might ultimately result in renal impairment [1-3]. This impairment is caused by the effect of both ischemia and reperfusion on different renal cells. Ischemia results in damage of various parts of the cell whereas restoration of blood further accentuates this injury which leads to release of oxygen free species and other cytokines and mediators of renal injury [1, 3, 4].

Ischemia-reperfusion injury is an invariable consequence of many conditions including nephron sparing renal surgery, trauma and transplantation [1, 5]. The number of nephron sparing renal surgery is increasing worldwide due to the higher detection rate of incidental small renal tumors which are now managed by nephron sparing surgery which often require clamping of renal vessels [6, 7]. Similarly, the number of live donor kidney transplantation is also increasing in several countries due to the shortage of cadaveric kidneys [8]. Both types of surgery often result in IRI and hence the growing need to search for medications which could protect the kidneys subjected to this type of injury.

Recently, there has been a growing interest in using natural phytochemical compounds as treatment alternatives in several conditions including renal diseases. This is due to their relatively low toxicity, price and availability. Indeed, it has been estimated that at least 25% of the drugs used over the past few decades, were directly derived from plants and another approximately 25% were chemically altered natural products [9]. Thymoquinone is one of these compounds. It is the main active ingredient of

Nigella sativa, commonly known as black cumin or black seed, an annual flowering plant native to some areas such the Mediterranean countries [10]. Since its first extraction in 1963 [11], thymoquinone has been shown to act as a potent free radical and superoxide scavenger [12-14]. In addition, thymoquinone has been shown to have an anti-inflammatory effect by inhibiting the exaggerated production of several cytokines and growth mediators [15-21]. The therapeutic effects of thymoquinone have been investigated in several conditions including renal conditions [13, 22-25]. Only limited number of studies has investigated the effect of thymoquinone in IRI [12, 13]. In both studies, serum creatinine was used as a rough estimate of renal function [26] and no attempt was made to study specific and more precise hemodynamic renal functions such the glomerular filtration rate and renal blood flow using more specific methodologies. Furthermore, the effect of thymoquinone on the IRI-induced renal tubular functions has not been investigated. Thus, the aim of this study was to investigate the effect of thymoquinone on various specific hemodynamic and tubular functions following IRI. Further, the effect of thymoquinone on some of the markers of renal injury and pro-inflammatory and pro-fibrotic cytokines has been studied in a trial to understand the effect of thymoquinone in this type of injury.

Materials and methods

Studies were performed in male Wistar rats weighing 211-237 g at the time of IRI. Rats were housed in standard cages and kept in a 12-hour light-dark cycle at 20°C. They were fed a standard rat chow and had free access to water. Animals were fasted for 12 hours before the experimental procedures but had water *ad libitum*. The experimental protocol was approved by the local animal research ethics committee.

Ischemia-reperfusion injury

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). The left renal artery was then exposed via a flank incision and occluded using microvascular non-traumatic bulldog clamp. Following a warm ischemia of 35 min, the microvascular clamp was removed to allow reperfusion. At the end, the wound was closed in layers.

Thymoquinone/vehicle administration

Thymoquinone (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.25 ml of corn oil (vehicle) and administered by oral gavage immediately after preparation as single daily dose of 10 mg/kg. The dose of thymoquinone used in this study was similar to that used in other studies in rat models [12, 13]. Control animals received only 0.25 ml of the vehicle, corn oil. In the two groups, treatment was commenced 4 days before IRI and continued 6 days thereafter until the time of the terminal experiment.

Experimental groups

Animals were divided into two groups: (1) Group-Vx (n=15): Rats underwent left renal ischemia and received only the vehicle corn oil. (2) Group-TQ (n=15): Rats underwent left renal ischemia and received thymoquinone.

Surgical procedure in the terminal experiment

All rats underwent terminal experiment six days following IRI. Animals were anaesthetised with pentobarbital sodium (45 mg/kg, intraperitoneally; Sigma Life Science, St Louis, USA) and the trachea was cannulated. The right femoral vein was then cannulated with polyethylene tubing (PE-50) and anaesthesia was maintained by a continuous infusion of pentobarbital sodium (15 mg/kg/hr) and a sustaining infusion of 0.9% saline was established at a rate of 50 µl/min using an infusion pump. The left femoral artery was cannulated with similar tubing used in the femoral vein and the tip of the cannula was positioned just below the level of the left renal artery. The cannula was connected to a pressure transducer (Memscap, Skoppum, Norway). The blood pressure signal was amplified using a bridge Amp (ADInstruments, Castle Hill, Australia), digitised using Power Lab 4/30 and Lab Chart version 6 software (ADInstruments, Australia) and displayed on a computer screen. The arterial cannula was also used to obtain blood samples throughout the procedure as required. Both kidneys were exposed through a midline abdominal incision and the upper ureters were cannulated with polyethylene tubing (PE-10) for the collection of

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

Gene	Gene bank reference		5'-3' Sequence
KIM-1	NM_173149.2	Forward	GCCTGGAATAATCACACTGTAAG
		Reverse	GCAACGGACATGCCAACATAG
		Probe	d FAM-TCCCTTTGAGGAAGCCGCAGA-BHQ-1
NGAL	NM_130741.1	Forward	CTGTTCCCACCGACCAATGC
		Reverse	CCACTGCACATCCCAGTCA
		Probe	d FAM-TGACAACTGAACAGACGGTGAGCG-BHQ-1
TNF-α	NM_012675.3	Forward	GGCTCCCTCTCATCAGTTCCAT
		Reverse	CGCTTGGTGGTTTGCTACG
		Probe	d FAM-CCCAGACCCTCACACTCAGATCATC -BHQ-1
TGF-β1	NM_021578.2	Forward	GTGGCTGAACCAAGGAGACG
		Reverse	CGTGGAGTACATTATCTTTGCTGTC
		Probe	dFAM-ACAGGGCTTTCGCTTCAGTGCTC-BHQ-1
PAI-1	NM_134432.2	Forward	GGCACAATCCAACAGAGACAA
		Reverse	GGCTTCTCATCCCACTCTCAAG
		Probe	d FAM-CCTCTTCATGGGCCAGCTGATGG-BHQ-1

urine into pre-weighed micro-capped tubes. The urine volume was determined gravimetrically.

On completion of surgery, the sustaining infusion of 0.9% saline was replaced by one 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) (0.4% w/v) in 0.9% saline. A priming dose of 2 ml of the same solution was infused over 2 minutes. Animals were allowed 45 minutes to equilibrate before being subjected to the experimental protocol.

Experimental protocol and assays

The experimental protocol consisted of two 20-minute clearance periods. Arterial blood samples (0.4 ml) taken at the beginning and end of the clearance periods were immediately centrifuged. Plasma samples (125 µl) were frozen to be assayed later. The plasma was replaced by an equal volume of saline and the erythrocytes were re-suspended by gentle vortexing and returned to the animal. The hematocrit was determined. Finally, after euthanizing the animals, the kidneys were removed, weighed and prepared for gene expression analysis (*vide infra*).

Urine and plasma samples were assayed for sodium level using a flame photometer (Corning, Halstead, Essex, England). Glomerular filtration rate (GFR) was estimated from the clearance of inulin. Renal blood flow (RBF) was calculated using the formula [RBF=ERPF/(1-hematocrit)], where the PAH clearance was used to estimate ERPF (effective renal plasma flow). The values of GFR, 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

The middle part of each kidney was excised, immediately snap-frozen in liquid nitrogen and stored at -80°C for a later measurement of gene expression of two of the markers of acute kidney injury (kidney injury molecule-1 (KIM1), neutrophil gelatinase-associated lipocalin (NGAL). We also measured the gene expressions of the tumour necrosis factor-alpha (TNF-α) which is a pro-inflammatory cytokine and transforming growth factor-β (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1) which are pro-fibrotic cytokines.

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

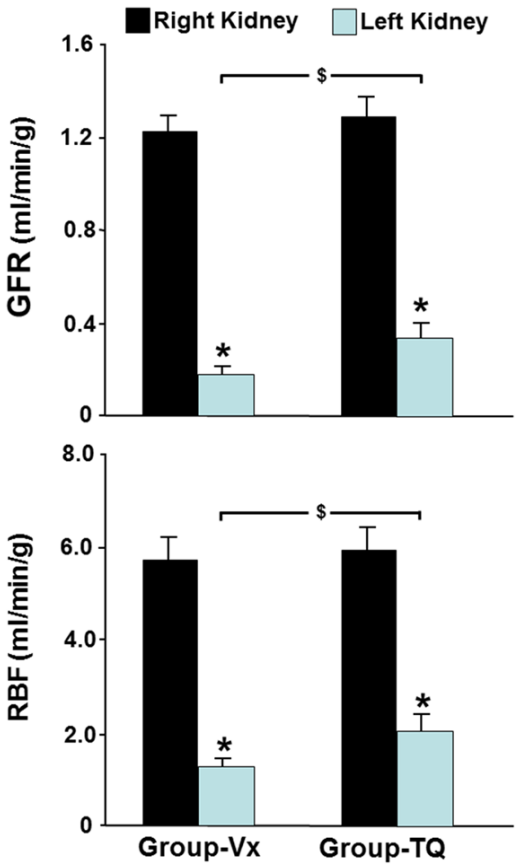

Figure 1. The glomerular filtration rate (GFR) and renal blood flow (RBF) in the right and left kidneys in Group-Vx and Group-TQ following left renal ischemia. Values represent mean ± SEM. *indicates statistical significance between the right and left kidney within the same group whereas $indicates statistical significance between the left ischemic kidneys in both groups.

cDNAs were prepared in duplicates from 2.0 µg of the extracted RNA with GoScript™ Reverse Transcriptase (Promega Corporation, Wisconsin, USA) in the presence of RNasin® Plus RNase inhibitor (Promega Corporation, Wisconsin, USA) according to manufacturer protocols. Prepared cDNA was used as a template for the relative gene expression analysis by real time PCR using TaqMan® chemistry on Applied Biosystems® 7500 Real-Time PCR instrument (Applied Biosystems, CA, USA). The reaction mixture consisted of 75 ng cDNA, TaqMan® Universal Master Mix (Applied Biosystems, CA, USA), 0.6 µM of forward and reverse primers and 0.25 µM of the fluorescent probes (Biosearch Technologies, Inc., CA, USA). Sequences 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-exon junction within their respective gene. Ribosomal protein lateral stalk subunit P0 Rplp0 (coding for 60S acidic ribosomal protein P0) was used as the endogenous control gene for normalization between samples. Calculated CT values were used to estimate changes in gene expression of target genes using delta-delta CT formula.

The results were expressed as the mean fold change of gene expression in the left ischemic kidney in the Group-TQ compared to Group-Vx.

Statistical analysis

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

Results

The mean arterial blood pressure and heart rate in Group-Vx and Group-TQ were similar (117±4 vs. 116±4 and 450±7 vs. 448±6, P=0.8 for both).

Glomerular and tubular functions

In Group-Vx, left RBF, six days following IRI, was 22% of the right RBF (1.27±0.21 vs. 5.77±0.54, P=0.0001). Similarly, left GFR was 15% that of the right GFR (0.18±0.03 vs. 1.22±0.07, P=0.0001) (**Figure 1**). With the decrease in both RBF and GFR, the FE_{Na} in the left kidney was significantly higher than the right kidney (2.40±0.35 vs. 0.59±0.08, P= 0.0001). This was associated with an increase in both the UV and $U_{Na}V$ in the left kidney (21.5±3.1 vs. 13.0±2.6, P=0.03 and 4.7±0.7 vs. 2.2±0.4, P=0.003, respectively (**Figure 2**).

In Group-TQ which received thymoquinone, the left RBF was 34% of the right RBF (2.02±0.39 vs. 6.00±0.55, P=0.0001) and the left renal GFR was 26% of the right GFR (0.33±0.08 vs. 1.29±0.08, P=0.0001) (**Figure 1**). As shown in

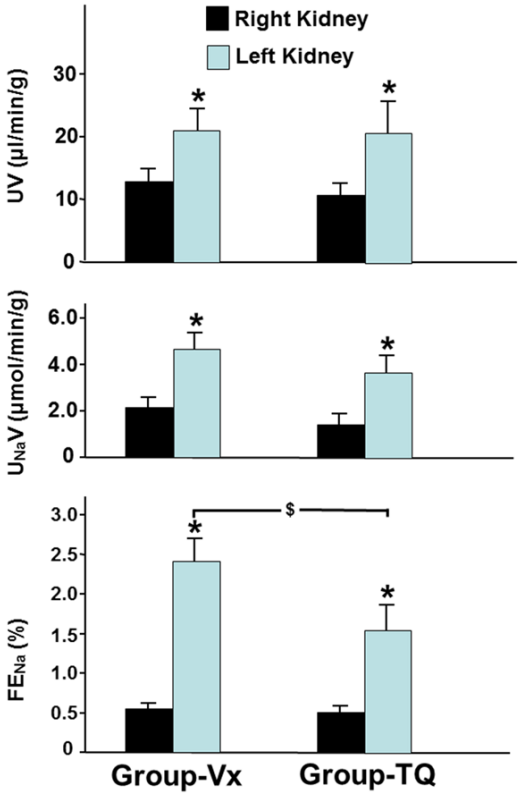

Figure 2. The tubular functional parameters including urine volume (UV), urinary sodium ($U_{Na}V$) and fractional excretion of sodium (FE_{Na}) in both kidneys in Group-Vx and Group-TQ following left renal ischemia. Values represent mean ± SEM. *indicates statistical significance between the right and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

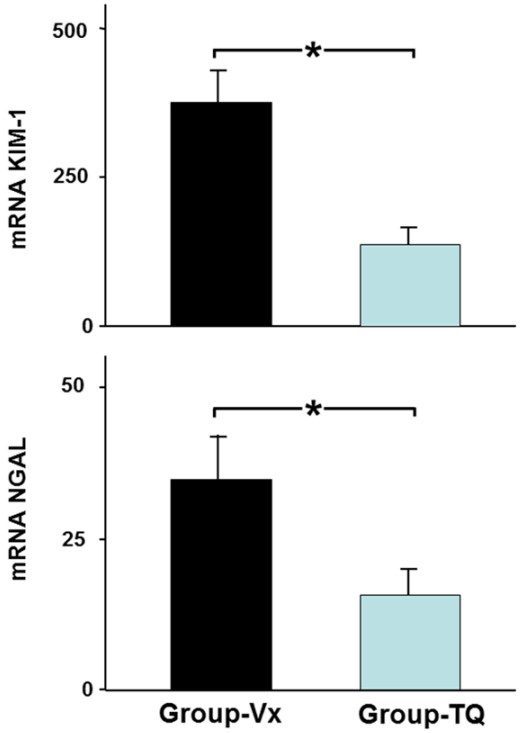

Figure 3. The expression of two of the markers of acute renal injury KIM-1 and NGAL in both groups. The results are expressed as the mean fold changes of gene expression in the left ischemic kidney in the Group-TQ compared to Group-Vx. Values represent mean ± SEM. *indicates statistical significance between groups.

Figure 2, the FE_{Na} of the left kidney were higher than that of the right control kidney (1.59±0.28 vs. 0.52±0.09, P=0.001). However, the UV and $U_{Na}V$ of the left kidney were lower than those of the right kidney (10.1±1.7 vs. 20.6±5.5, P=0.04 and 1.4±0.4 vs. 3.6±0.8, P=0.001, respectively).

When Group-TQ was compared to Group-Vx, all variables in the right non-ischemic kidneys in both groups were similar (P>0.05 for all variables). However, when the left ischemic kidneys in the two groups were compared, both the left RBF and GFR were higher in Group-TQ (2.02±0.39 vs. 1.27±0.21, P=0.04 and 0.33±0.08 vs. 0.18±0.03, P=0.03, respectively) (**Figure 1**). As shown in **Figure 2**, thymoquinone improved the left renal FE_{Na} (1.59±0.28 vs. 2.40±0.35, P=0.04) (**Figure 2**). However, the UV and $U_{Na}V$ were not different among both groups (P>0.05 for both variables).

Gene expression analysis results

As demonstrated in **Figure 3**, in Group-Vx, there was 358±49 fold increase in the expression of KIM-1 in the left ischemic kidney compared to the right control kidney, whereas in Group-TQ, there was only 143±20 fold increase (P=0.004). Similarly, the left to right kidney expression of NGAL was lower in Group-TQ (16±3 vs. 34±6 P=0.048) (**Figure 3**). Similar findings were obtained for TNF-α, TGF-β1 and PAI-1, (1.1±0.2 vs. 2.8±0.4, P=0.02, 1.6±0.1 vs. 2.8±0.1, P=0.0001 and 2.4±0.3 vs. 5.8±1.0, P=0.02, respectively) (**Figure 4**).

Discussion

In the current study, we have demonstrated for the first time that administration of thymoquinone prior to and following IRI has specifically

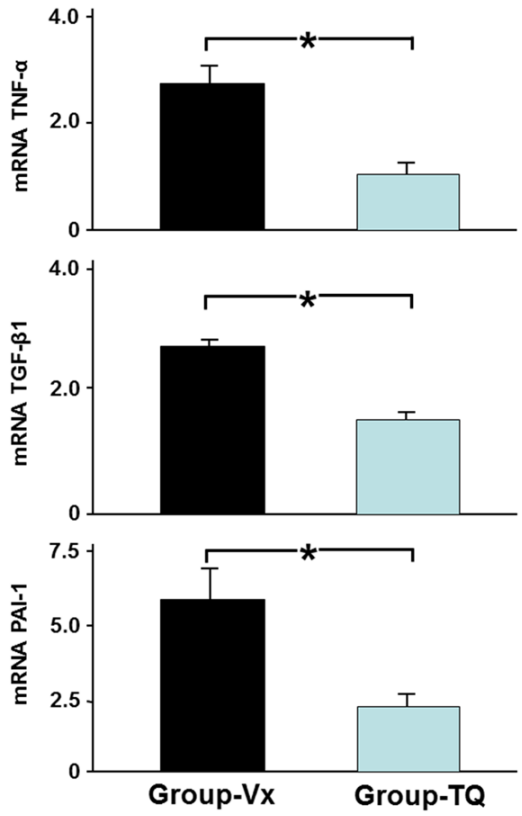

Figure 4. The expression of the pro-inflammatory and pro-fibrotic cytokines TNF-α, TGF-β1 and PAI-1 in both groups. The results are expressed as the mean fold changes of gene expression in the left ischemic kidney in the Group-TQ compared to Group-Vx. Values represent mean ± SEM. *indicates statistical significance between groups.

resulted in a significant improvement in the hemodynamic and tubular renal functional parameters and in attenuation of the gene expression of some of the pro-inflammatory and pro-fibrotic cytokines, namely TNF-α, TGF-β1 and PAI.

The pathogenesis of the IRI-induced renal damage is caused by both ischemia and reperfusion and appears to be multifactorial. It includes hypoxia, excessive production of reactive oxygen species and inflammatory response characterized by increased production of several cytokines such as pro-inflammatory and pro-fibrotic cytokines including TNF-α, TGF-β1 and PAI [1, 3, 4, 27-29].

Using different experimental models, the protective effects of thymoquinone have been shown to be due to several properties including its antioxidant and free radical scavenging activity [12-14]. In addition, thymoquinone has the ability to inhibit the production of some inflammatory mediators [15-21]. This property, although demonstrated in other models [15-21], has not been previously shown in IRI. The present study was the first to demonstrate this activity in IRI providing other evidence of the protective effect of thymoquinone in different organs and models. In the present study, we investigated the effect of thymoquinone on TNF-α, TGF-β1 and PAI in IRI. TNF-α is a pro-inflammatory cytokine that is produced by several cells including renal cells [30]. TGF-β is a pro-fibrotic cytokine which stimulates renal cells to produce extracellular matrix proteins leading to glomerulosclerosis and tubulointerstitial fibrosis [29, 31]. PAI-1 is also a pro-fibrotic cytokine as it is considered as the major inhibitor of fibrinolysis [32, 33]. All these cytokines get up-regulated in renal diseases [27-29] and the protective effect of thymoquinone on these cytokines in IRI appears to be similar to its action in other conditions.

These effects of thymoquinone probably account, at least partially, to the improvement in renal functional parameters observed in the present study. The current data indicates that thymoquinone does not only affect the hemodynamic renal functional parameters namely GFR and RBF but also the tubular renal functions. In this model, the difference in the RBF and GFR between the left kidneys in both group was not highly significant as shown by the *P* value of 0.04 and 0.03, respectively. Nevertheless, it has reached statistical significance with this number of animals. This effect was obviously weaker than the effect observed on the tubular functions as thymoquinone significantly reduced the increase in FE_{Na} observed in the ischemic kidney and hence improved its' ability to concentrate urine. This effect of thymoquinone has not been reported previously. The lack of significant effect of thymoquinone on total UV and $U_{Na}V$ observed in the current study, is probably due to the fact that these parameters are not determined only by the FE_{Na} but also by other parameters such as the GFR. Since thymoquinone has caused a decrease in FE_{Na} but an increase in GFR, the net result would be a lack of significant change in UV and $U_{Na}V$.

In addition to ameliorating the alterations in renal functional parameters, thymoquinone de-

creased the expression of some of the markers of acute renal injury such as KIM-1 and NGAL. KIM-1 has been shown to be strongly expressed and released by injured proximal tubular epithelial cells [34] whereas NGAL is synthesized in the thick ascending limb of Henle's loop and collecting ducts [35]. Thus, the results of this study indicate that thymoquinone has affected different parts of renal tubules accounting for the improvement in the renal concentration ability.

The ischemic model used in this study is similar to the clinical scenario of a transient ischemia seen in conditions such as nephron sparing renal surgery, aortic clamping and renal transplantation. Since the overall rate of performing these types of surgery is increasing worldwide (*vide supra*), the protective effects of thymoquinone shown in the present study might be of clinical interest and such patients might benefit from taking this agent or Nigella sativa which has a high content of this compound. However, further clinical studies are required to extrapolate the results to humans.

In conclusion, the administration of thymoquinone before during and after IRI appears to have ameliorated the IRI effect on the hemodynamic and tubular renal functional parameters as well as the expression of some of markers of acute renal injury. It has also attenuated the expression of some of pro-inflammatory and pro-fibrotic cytokines indicating a renoprotective effect of this agent in ischemia-reperfusion injury and a potential clinical implication.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

IRI, ischemia-reperfusion injury; GFR, glomerular filtration rate; RBF, renal blood flow; UV, urine volume; $U_{Na}V$, urinary sodium; FE_{Na}, fractional excretion of sodium; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; TNF-α, tumour necrosis factor-alpha; TGF-β1, transforming growth factor-β1; PAI-1, plasminogen activator inhibitor-1; PCR, polymerase chain reaction; SEM, standard error of the mean.

Address correspondence to: Fayez T Hammad, Department of Surgery, College of Medicine & Health Sciences, PO Box 17666, Al Ain, United Arab Emirates. Tel: 00971 50 4880021; 00971 3 7137 590; Fax: 00971 3 7672067; E-mail: fayezhammad@hotmail.com; fayezh@uaeu.ac.ae

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