

Original Article

The effect of epigallocatechin-3-gallate on the renal dysfunction in the obstructed kidney 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: Epigallocatechin-3-gallate (EGCG) is the most active catechin polyphenol extracted from the green tea. EGCG has protective effects in various renal and non-renal conditions. The aim of this study was to investigate the effect of EGCG on the alterations in renal functional parameters following reversible unilateral ureteral obstruction (UUO) in the rat. Methods: Wistar rats underwent reversible left UUO for 72 hours. Group-EGCG (n=10) received intraperitoneal 50 mg/kg/day of EGCG whereas Group-Vx (n=10) had only normal saline. Five days post UUO reversal, renal functions of both kidneys were measured using clearance techniques and the gene expression of some of kidney injury markers (KIM-1 and NGL) and the pro-inflammatory mediator (TNF- α) were determined using real time PCR. Results: Renal blood flow, glomerular filtration rate, urine volume and urinary sodium excretion were still altered 5 days post-UUO reversal. Fractional sodium excretion had returned to baseline values by that time. EGCG did not significantly affect any of the renal functional parameters of the obstructed kidney ($P>0.05$ for all). However, it significantly decreased the gene expressions of KIM-1, NGAL and TNF- α in the left obstructed kidney in Group-EGCG compared to Group-Vx (28 ± 27 vs. 286 ± 107 , 1.1 ± 0.2 vs. 10.9 ± 4.3 , and 0.8 ± 0.1 vs. 1.5 ± 0.2 , $P<0.05$ for all). Conclusion: EGCG appears to have no significant protective effect on the haemodynamic or tubular glomerular functions when measured as early as five days post reversal of UUO despite the attenuation of some of the kidney injury markers and pro-inflammatory mediators.

Keywords: Epigallocatechin-3-gallate, ureteral obstruction, renal functions

Introduction

Ureteral obstruction (UO) is a common clinical problem worldwide and caused by conditions such as urinary lithiasis. It usually causes pain and if prolonged may lead to renal impairment which is associated with alterations in several injury-induced mediators [1-4]. There has been an ongoing search for therapeutic agents to attenuate this damage and recently, there has been a growing interest in natural phytochemical compounds which are used as treatment alternatives in many conditions including renal diseases. This is probably due to their relatively low toxicity, price and wide availability. At least 25% of the therapeutic agents consumed over the past few decades has been estimated to be directly derived from plants and another approximately 25% were chemically altered natural products [5].

Green tea is believed to be one of the healthiest natural drinks worldwide. It contains various catechin polyphenols which have anti-oxidative, anti-inflammatory, anti-cancerous and anti-infective properties [6-8]. Epigallocatechin-3-gallate (EGCG) is the most abundant and most active catechin polyphenol extracted from green tea [9]. Previous studies have evaluated the protective effect of epigallocatechin-3-gallate in various renal [10, 11] and non-renal [7-9, 12, 13] conditions. Few studies have investigated the effect of EGCG on the renal dysfunction following UO [14-17]. These studies mainly addressed the effect of EGCG on the alterations in some of the oxidative and inflammatory mediators and the effect of EGCG on the clinically more relevant renal functional parameters was only addressed by one study [15]. In this study, serum creatinine and blood urea nitrogen were used as indicators of renal functions. Such indicators have been shown to be imprecise and

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form a rough estimate of renal functions [18]. No attempt was made to study more specific and precise hemodynamic renal functions such as the glomerular filtration rate and renal blood flow using more specific methodologies. Furthermore, the effect of EGCG on the UO-induced renal tubular functions has not been investigated yet. Therefore, the aim of this study was to investigate the effect of EGCG on the alterations in renal hemodynamic and tubular functions in a model of reversible unilateral ureteral obstruction (UUO) in the rat.

Materials and methods

Studies were performed in male Wistar rats weighing 197-213 gm at the time of UUO. 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.

Ureteral occlusion and reversal

Under aseptic conditions, animals were anaesthetized with intraperitoneal injection of ketamine hydrochloride (80 mg/kg, Pantex Holland B.V., Holland) and xylazine Hydrochloride (8 mg/kg, Troy Laboratory PTY Limited, NSW, Australia). The left ureter was exposed via midline abdominal incision and obstructed by placing a 3-4 mm length of bisected PVC tubing (0.58 mm internal diameter) around the midureter as described previously [1]. The ureter was then occluded by constricting the tubing with a 4-0 silk suture. At the end, the wound was closed in layers.

The reversal of UUO was performed 72 hours later using similar anesthesia. Using the same incision, the obstructing tube was identified and removed with the assistance of dissecting microscope. Full release of the obstruction was confirmed by observation of free flow of urine across the site of obstruction. The wound was then closed in layers.

Epigallocatechin-3-gallate administration

(-)-Epigallocatechin-3-gallate (EGCG) (Cayman-pharma, Czech Republic) was dissolved in

saline and given as single intraperitoneal injection of 50 mg/kg/day for a total of 9 days. Treatment was commenced two days before UUO surgery, throughout the 72 hours obstruction period and continued 5 days after the UUO reversal until the time of terminal experiment 5 days post reversal of UUO.

Experimental groups

Animals were divided into two groups: 1. Group-Vx (n=10): Rats underwent left UUO and received only intraperitoneal normal saline. 2. Group-EGCG (n=10): Rats underwent left UUO and received EGCG.

Surgical procedure in the terminal experiment

All rats underwent terminal experiment five days following UUO reversal. Animals were anaesthetized 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 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 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%

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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 |

saline. A priming dose of 2 ml of the same solution was infused over 2 minutes. Animals were allowed 75 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 μ l) 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). PAH is filtered by the glomeruli and is actively secreted by the proximal tubules, therefore, its extraction ratio is high when infused at a rate which maintains the plasma concentration below the transport maximum [19, 20]. It is ideally suited for measurement of ERPF since it has a high clearance is essentially nontoxic at the plasma concentrations reached with recommended doses, and its analytical determination is relatively simple and accurate. The val-

ues 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 (six rats in each group) was excised, im-

mediately snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$ for a later measurement of gene expression of two of the markers of acute kidney injury (kidney injury molecule-1 (KIM-1), 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.

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 2.0 μ g 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 μ M of forward and reverse primers and 0.25 μ M 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 Real Time Design[™] 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

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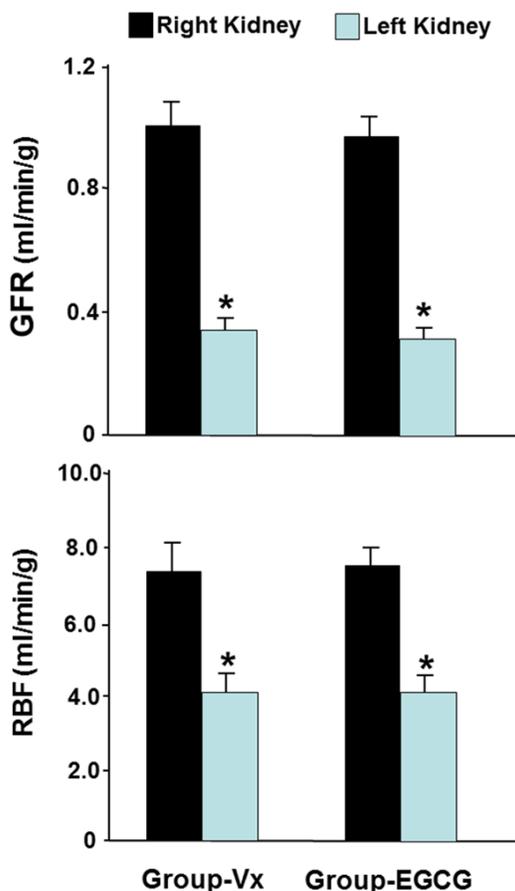


Figure 1. The glomerular filtration rate (GFR) and renal blood flow (RBF) in the right and left kidneys in Group-Vx and Group-EGCG following reversal of left ureteral obstruction. Values represent mean±SEM. *indicates statistical significance between the right and left kidney within the same group.

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 obstructed kidney in Group-EGCG 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-1 and Group-2 were similar (117±4 vs. 115±3 and 453±8 vs. 447±6, *P*>0.05 for both).

Glomerular and tubular functions

In Group-Vx, left RBF, five days following UUU reversal, was 56% of the right RBF (4.11±0.53 vs. 7.35±0.81, *P*<0.01). The left GFR was 35% that of the right GFR (0.35±0.03 vs. 0.99±0.07, *P*<0.01) (**Figure 1**). With the decrease in both RBF and GFR, the fractional excretion of sodium (FE_{Na}) had recovered by this time (0.7±0.1 vs. 0.6±0.2, *P*>0.05) (**Figure 2**). This was associated with a decrease in both the urine volume (UV) and urinary sodium excretion ($U_{Na}V$) in the left kidney (10.1±2.0 vs. 43.8±6.8, *P*<0.001 and 2.4±0.4 vs. 8.3±1.0, *P*<0.001, respectively (**Figure 2**).

In Group-EGCG which received Epigallocatechin, the left RBF was 53% of the right RBF (4.10±0.34 vs. 7.77±0.56, *P*<0.001) and the left renal GFR was 33% of the right GFR (0.32±0.03 vs. 0.96±0.07, *P*<0.001) (**Figure 1**). As shown in **Figure 2**, the FE_{Na} of the left kidney was similar to the right control kidney (0.7±0.1 vs. 0.7±0.1 (*P*>0.05). However, the UV and $U_{Na}V$ of the left kidney were lower than those of the right kidney (11.7±3.0 vs. 38.9±3.1, *P*<0.001 and 2.2±0.5 vs. 8.3±0.8, *P*<0.001, respectively).

When Group-EGCG was compared to Group-Vx, all variables in the right non-obstructed kidneys in both groups were similar (*P*>0.05 for all variables). Similarly, when the left obstructed kidneys in the two groups were compared, all variables were similar (*P*>0.05 for all variables) (**Figures 1 and 2**).

Gene expression analysis results

As demonstrated in **Figure 3**, in Group-Vx, there was 286±107 fold increase in the expression of KIM-1 in the left obstructed kidney compared to the right control kidney, whereas in Group-EGCG, there was only 28±27 fold increase (*P*<0.05). Similarly, the left to right kidney

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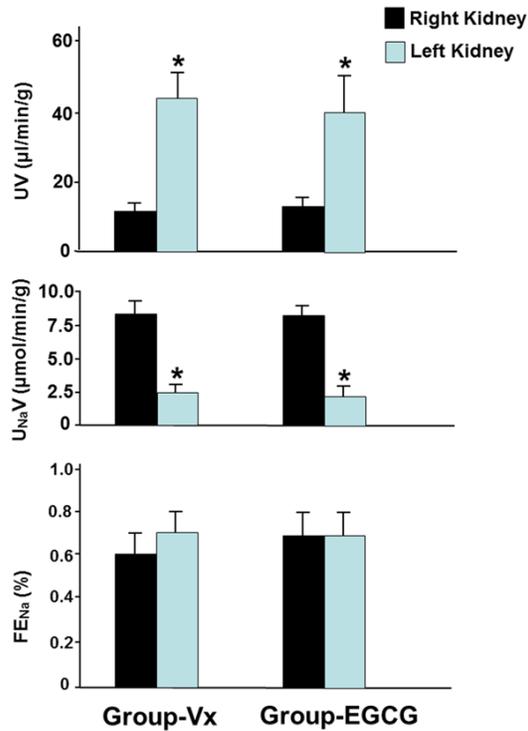


Figure 2. The tubular functional parameters including urine volume (UV), urinary sodium (U_{NaV}) and fractional excretion of sodium (FE_{Na}) in both kidneys in Group-Vx and Group-EGCG following reversal of left ureteral obstruction. Values represent mean \pm SEM. *indicates statistical significance between the right and left kidney within the same group.

expression of NGAL was lower in Group-EGCG (1.1 ± 0.2 vs. 10.9 ± 4.3 , $P<0.05$) (Figure 3). As shown in Figure 4, similar findings were obtained for TNF- α (0.8 ± 0.1 vs. 1.5 ± 0.2 , $P<0.05$).

Discussion

In this model of three days reversible UUO, we have shown that EGCG has attenuated the alterations in some of the obstruction-induced renal injury markers such as KIM-1 and NGAL and some of the inflammatory mediators such as TNF- α . However, EGCG did not significantly affect any of the hemodynamic or tubular renal functional parameters such as the RBF, GFR or sodium excretion.

In the current study, EGCG appears to have some protective renal effects as indicated by the attenuation of the UO-induced increase in the TNF- α which was shown to be one of the acute inflammatory and pro-apoptotic media-

tors in UO [21-23]. For instance, both the serum level [24] and mRNA of TNF- α [25] were shown to rise sharply soon after the obstruction of the ureter. This acute increase in the production of TNF- α , rendered this cytokine an appropriate marker in the current experiment in which the terminal experiment was performed five days following the reversal of obstruction.

This attenuation in TNF- α by EGCG has been shown by other studies which used similar models of UO [15, 16]. EGCG has also attenuated other pro-inflammatory mediators and oxidative stress markers [14-17]. In addition, we have also demonstrated for the first time, that EGCG has also ameliorated the rise in specific markers of renal injury namely KIM-1 and NGAL. KIM-1 has been shown to be strongly expressed and released by injured proximal tubular epithelial cells [26] whereas NGAL is synthesized in the thick ascending limb of Henle's loop and collecting ducts [27]. Thus, the results of this study indicate that EGCG has affected different parts of renal tubules.

In the current study, despite the attenuation of these markers and mediators, EGCG did not significantly affect the renal hemodynamic or tubular parameters which were similar in the two groups. This lack of protective effect is unlikely to be due to bioavailability issues because similar protective dose was used in the rat by other investigators in both renal [28] and non-renal conditions [29-32]; further, the attenuation of the alterations in the renal injury markers and the TNF- α observed in Group-EGCG, indicates that the drug was bioavailable. This lack of improvement in the clinically more relevant renal functional parameters despite the improvement in the injury-induced mediators has been reported previously with other therapeutic agents in UO [33] and this might apply to other therapeutic agents in other conditions. Indeed, the literature is full of studies which show the beneficial protective effects of many therapeutic agents on injury-induced markers in various organs. The vast majority of these studies, however, did not further investigate the effect of these therapeutic agents on the more relevant clinical parameters.

In the current study, it is difficult to determine the exact reason for the lack of the improvement in the renal functions. This could be due to the severity of the injury induced by the 3

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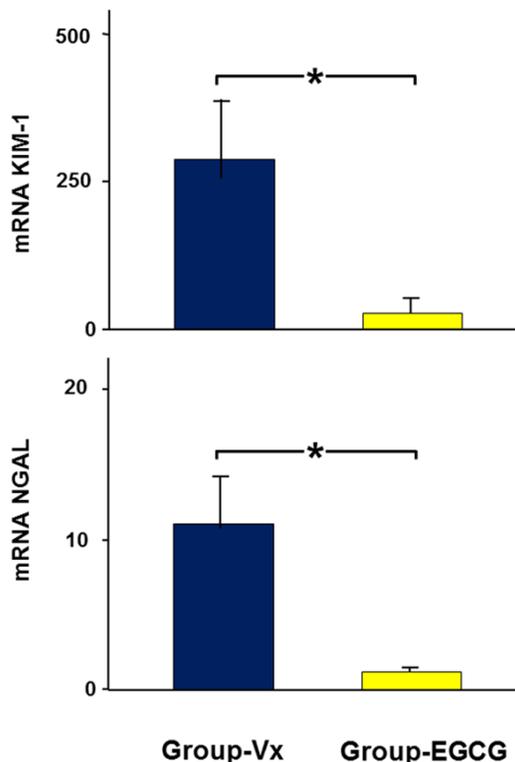


Figure 3. The expression of two of the markers of acute renal injury KIM-1 and NGAL in both groups. The results are expressed as the mean fold changes of gene expression in the left obstructed kidney in the Group-EGCG compared to Group-Vx. Values represent mean±SEM. *indicates statistical significance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

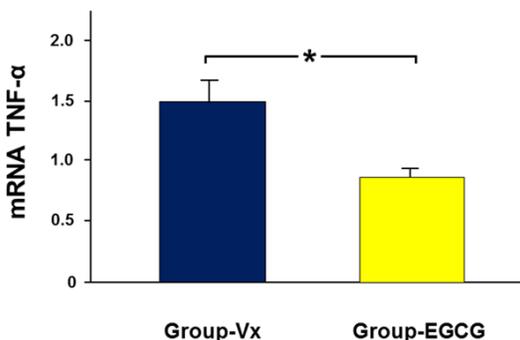


Figure 4. The expression of the pro-inflammatory cytokine TNF- α in both groups. The results are expressed as the mean fold changes of gene expression in the left obstructed kidney in the Group-EGCG compared to Group-Vx. Values represent mean±SEM. *indicates statistical significance between groups.

days UUO and a weakness in the efficacy of EGCG which might be due to its weak effect on the changes in the main mediators involved in

the early alterations observed in UO such as angiotensin II, prostaglandins, and endothelins [1-4]. Alternatively, it could be that it takes longer time for the effect of EGCG to be shown as improvements in clinical parameters. In support of the later, Ito et al and others have shown that there was a progression of renal disease despite the initial improvement in renal functional parameters following the reversal of obstruction [34, 35]. Therefore, it is possible that the initial attenuation in renal injury markers and inflammatory mediators would result in a better outcome in the long term. Certainly, further studies are required to explore this issue.

This model of reversible UO was selected in the current study due to its similarity to the common clinical scenario of a transiently obstructing ureteral stone. The validity of the model was demonstrated by the significant drop in the GFR and RBF in the obstructed kidney even 5 days after the reversal of obstruction, similar to what was reported in other studies [36, 37].

In conclusion, the administration of epigallocatechin-3-gallate before, during and after reversal of relatively long period of ureteral obstruction appears to have no significant protective effect on the renal haemodynamic or tubular functional parameters when measured five days following the reversal of obstruction despite the amelioration in some of the renal injury markers and pro-inflammatory mediators.

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Disclosure of conflict of interest

None.

Abbreviations

UO, ureteral obstruction; UUO, unilateral ureteral obstruction; 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; PCR,

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polymerase chain reaction; SEM, standard error of the mean.

Address correspondence to: Dr. 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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