

Effect of thymoquinone on hepatorenal dysfunction and alteration of CYP3A1 and spermidine/spermine N-1-acetyl-transferase gene expression induced by renal ischaemia–reperfusion in rats

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Abstract

Objectives Renal ischaemia–reperfusion (I/R) is a well-characterised model of acute renal failure that causes both local and remote organ injury. The aim of this work was to investigate the effect of thymoquinone, the main constituent of the volatile oil extracted from *Nigella sativa* seeds, on renal and hepatic changes after renal ischaemia–reperfusion.

Methods Male Sprague-Dawley rats were divided into sham I/R vehicle-treated groups, and I/R thymoquinone-treated groups. Thymoquinone (10 mg/kg, p.o.) was administered for ten consecutive days to the I/R thymoquinone group before injury. I/R and I/R thymoquinone groups were subjected to 30-min ischaemia followed by 4-h reperfusion.

Key findings I/R resulted in a significant increase in malondialdehyde (MDA) level and decreases in glutathione-S-transferase (GST) and superoxide dismutase (SOD) activity in liver and kidney tissues. Thymoquinone treatment caused the reversal of I/R-induced changes in MDA as well as GST and SOD activity. Moreover, I/R caused a significant rise in creatinine and alanine aminotransferase serum levels. CYP3A1 mRNA expression was induced significantly by I/R in both liver and kidney tissues compared with sham group. Thymoquinone reduced significantly this increase. I/R caused induction of mRNA expression of spermidine/spermine N-1-acetyl-transferase (SSAT), a catabolic enzyme that participates in polyamine metabolism, in liver and kidney tissues. Thymoquinone reduced SSAT mRNA expression significantly in liver and markedly in kidney.

Conclusions These findings suggested that thymoquinone protected against renal I/R-induced damage through an antioxidant mechanism as well as the decrease of CYP3A1 and SSAT gene expression.

Keywords CYP3A1; oxidative stress; renal ischaemia–reperfusion; spermidine/spermine N-1-acetyl-transferase; thymoquinone

Introduction

Renal ischaemia–reperfusion (I/R) occurs in many clinical scenarios, including trauma, elective surgery, and transplantation. Events initiated by this process can lead to inflammation in the kidneys, culminating in local injury as well as distant organ dysfunction.^[1]

Previous reports indicated that renal I/R could lead to distant organ dysfunction in addition to inflammation in the renal tissues resulting in local tissue injury.^[2] Furthermore, the anti-oxidants superoxide dismutase (SOD), glutathione peroxidase, catalase and glutathione levels were all reduced in the liver after renal I/R injury in rabbits.^[3] It is commonly believed that organs remote to the site of I/R injury can be affected by circulating cytokines originating from these I/R injuries.^[4]

Multiple alterations in the liver after I/R in rat including alteration of metabolizing enzymes such as CYP3A have been found.^[1] CYP3A1 is responsible for metabolizing ciclosporin, a potent and effective immunosuppressive agent, which is widely used for the prevention of allograft rejection following tissue transplants.^[5]

Spermidine/spermine N-1-acetyl-transferase (SSAT) is a catabolic enzyme that participates in polyamine metabolism influencing cell proliferation, differentiation, death and survival. SSAT has been reported to be induced in some organs subjected to ischaemia–reperfusion and considered as a sensitive marker of injury.^[6]

Renal I/R injury is an important determinant of allograft survival after transplantation. Studies have shown that early I/R injury can lead to the initiation of inflammatory cascade, which may result in delayed graft function and decreased long-term renal allograft survival.^[7]

Oxygen free radicals, produced on reperfusion, play a critical role in the injury caused by ischaemia–reperfusion.^[8] Reactive oxygen species (ROS) lead to an inflammatory response and tissue damage by activating some mediators such as proinflammatory cytokines, chemokines and adhesion molecules. They can also directly damage cell components such as DNA, protein and lipids.^[9] Protection against reperfusion injury can be induced by assorted treatments including administration of antioxidants and anti-inflammatory drugs.^[10]

Nigella sativa has a protective effect against I/R injury to various organs.^[11] Moreover, it has been demonstrated that *N. sativa* can significantly prevent hepatotoxicity and might have protective effects against nephrotoxicity induced by either disease or chemicals.^[12,13]

Thymoquinone, the active constituent of *N. sativa* seeds, is a pharmacologically active quinone, which possesses several properties including analgesic and anti-inflammatory actions.^[14] It has been reported that thymoquinone prevented oxidative injury in in-vitro and in-vivo studies in rodents.^[15,16] Additional mechanisms of action may be related to inhibition of eicosanoid generation, namely thromboxane B₂ and leukotrienes B₄ (by inhibiting cyclooxygenase and 5-lipoxygenase, respectively), and membrane lipid peroxidation.^[17] Thymoquinone protected the kidney against ifosfamide-induced damage by preventing renal glutathione depletion and lipid peroxide accumulation.^[18] In addition, orally administered thymoquinone prevented cisplatin-induced nephrotoxicity in mice and rats as evidenced by significant reductions in serum urea, and by a significant improvement in kidney weight and creatinine clearance.^[19]

However, in spite of the substantial literature evidence on the antioxidant power of thymoquinone in various models of oxidative stress, the molecular mechanism of this pharmacological action needs further study.

In this work, we have investigated the possible effect of thymoquinone on kidney damage induced by bilateral renal I/R. For the first time, we have studied the effect of thymoquinone on the remote action of kidney I/R on the liver. We assessed the effect of thymoquinone on CYP3A1 and SSAT gene expression as well as antioxidant parameters.

Materials and Methods

Chemicals and drugs

Thymoquinone and all the chemicals were analytical grade purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Animal preparation and experimental design

Thirty six adult male Sprague-Dawley rats (The Nile Company for Pharmaceutical and Chemical Industries, Cairo, Egypt; 200–250 g) were housed in cages in a temperature-controlled (25 ± 1°C) environment. The rats had free access to pelleted food and purified drinking water was freely avail-

able. The animal experiments described below complied with the ethical principles and guidelines for the care and use of laboratory animals adopted by the National Egyptian Community. The Animal experimental protocols were approved by the Al-Azhar University Committee.

The rats were divided into three groups (12 animals each). Group I, sham operated group. Group II, renal I/R group, underwent 30-min ischaemia followed by 4-h reperfusion. This group received 10% Tween 20 solution orally by gavage for ten days before the operation. Group III, renal I/R group receiving thymoquinone. Thymoquinone was suspended in 10% Tween 20 solution and administered at a dose of 10 mg/kg of body weight orally by gavage for ten days before the operation. Surgery was performed under chloral hydrate (400 mg/kg, i.p) anaesthesia. A midline abdominal incision was performed to expose both renal pedicles. In group I, pedicles were isolated but not occluded. In groups II and III, both renal pedicles were occluded with suture for 30 min. Reperfusion was allowed by withdrawal of the occluding suture for 4 h.

At the end of reperfusion, blood samples were collected; liver and kidney were rapidly removed. Parts of both organs were snap frozen in liquid nitrogen and stored at –80°C for semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). The remaining tissues were homogenized for biochemical evaluation.

Liver and kidney function tests

Serum concentrations of alanine aminotransferase (ALT) and creatinine were measured as indicators of hepatic and renal injury, respectively, using standard diagnostic kits (Quimica Clinica Aplicada s.a., Amposta, Spain).

Determination of lipid peroxide level

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) level in the liver and kidney homogenates.^[20]

Evaluation of antioxidant enzyme activity

Determination of glutathione-S-transferase activity

The glutathione-S-transferase (GST) activity towards 1-chloro-2,4-dinitrobenzene in the presence of glutathione as co-substrate was examined spectrophotometrically at 25°C. The enzyme activity was determined by monitoring change in absorbance at 340 nm.^[21]

Determination of superoxide dismutase activity

SOD activity was determined by calculating the difference between auto-oxidation of pyrogallol alone and in the presence of homogenate that contained SOD.^[22]

Determination of total protein

The protein content was measured using bovine serum albumin as a standard.^[23]

Semiquantitative RT-PCR

Liver and kidney samples stored at –80° were used. Total cellular RNA was isolated by homogenizing tissues with Biozol extraction reagent (Flowgen Bioscience, Nottingham,

Table 1 Primer sequences

	Sense primer	Antisense primer
CYP3A1	5'GATGTTGAAATCAATGGTGTGT 3'	5'TTCAGAGGTATCTGTGTTCC 3'
SSAT	5' CTCCAAGAGGATGGCTTTGG 3'	5'ACTGGACAGATCCGAAGCAC 3'
GAPDH	5'GGTGAAGTCCGGTGTCAACGGATT 3'	5'GATGCCAAAGTTGTCATGGATGACC 3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSAT, spermidine/spermine N-1-acetyl-transferase.

Table 2 Effect of thymoquinone on lipid peroxidation, glutathione-S-transferase and superoxide dismutase activity after renal ischaemia–reperfusion

Groups	MDA (nmol/g tissue)		GST (nmol/min/mg protein)		SOD (U/mg protein)	
	Liver	Kidney	Liver	Kidney	Liver	Kidney
Sham	95.05 ± 5.91	102.53 ± 10.73	27.04 ± 2.8	20.94 ± 1.9	14.22 ± 1.4	14.01 ± 1.14
Renal I/R	356.92 ± 57.65 ^a	188.46 ± 28.21 ^a	14.49 ± 1.8 ^a	10.96 ± 1.4 ^a	7.85 ± 0.11 ^a	8.31 ± 0.72 ^a
Thymoquinone + I/R	135.38 ± 18.07 ^b	109.71 ± 14.83 ^b	23.17 ± 2.2 ^b	17.02 ± 1.8 ^b	11.93 ± 0.96 ^b	11.79 ± 0.66 ^b

Results are expressed as the mean ± SEM ($n = 10$ to 12) in each group. GST, glutathione-S-transferase; I/R, ischaemia–reperfusion; MDA, malondialdehyde; SOD, superoxide dismutase. ^a $P < 0.05$, significantly different from sham group. ^b $P < 0.05$, significantly different from renal I/R.

UK). Total RNA was extracted with chloroform and samples were centrifuged at 12 000g for 15 min at 4°C. The RNA was precipitated by isopropanol and the pellet was dissolved in 75% ethanol. RT-PCR One-step Kit (Jena Bioscience, Jena, Germany) was used for one-step reverse transcription and PCR. The cDNA was amplified using 30 pmol/30 µl reaction mixture volume of specific primers (Table 1) with an MJ Research thermal cycler (Ramsey, MN, USA). Reverse transcription was performed at 37°C for 60 min for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and at 48°C for 60 min for CYP3A1 and SSAT. This was followed by denaturation at 95°C for 5 min followed by 25 cycles. Each cycle consisted of 94°C for 45 s, 60°C for 60 s, and 72°C for 90 s for GAPDH, and 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s for CYP3A1 and SSAT. Final elongation was done at 72°C for 10 min. After amplification, the PCR products (5 µl) were separated on a 1.5% agarose gel containing 1 µg/ml ethidium bromide, and bands were visualized and photographed using ultraviolet transillumination. The size of each PCR product was determined by comparison with a standard DNA size marker. The relative levels of CYP3A1 and SSAT were normalised to GAPDH after semiquantitative assessment of gene expression using the Gel-pro analyser v4 program and expressed in arbitrary units.

Statistical analysis

Results are expressed as the mean ± SEM and the different groups were compared using one-way analysis of variance followed by Tukey-Kramer test for multiple comparisons.

Results

Effect of thymoquinone on oxidative stress induced by renal I/R

Renal I/R induced a significant rise in the level of MDA, an index for lipid peroxidation, in both liver and kidney tissues. Treatment with thymoquinone (10 mg/kg) decreased the MDA level in liver and kidney by 62.06 and 41.78%, respectively (Table 2).

Table 3 Effect of thymoquinone on creatinine and alanine aminotransferase serum level in rats

Groups	Creatinine (mg/dl)	ALT (IU/ml)
Sham	0.43 ± 0.18	43.57 ± 0.41
Renal I/R	1.18 ± 0.11 ^a	65.88 ± 8.79 ^a
Thymoquinone (10 mg/kg) + I/R	0.66 ± 0.11 ^b	46.5 ± 1.39 ^b

Results are expressed as the mean ± SEM ($n = 10$ to 12) in each group. ALT, alanine aminotransferase; I/R, ischaemia–reperfusion. ^a $P < 0.05$, significantly different from sham group. ^b $P < 0.05$, significantly different from renal I/R.

Compared with the sham group, the activity of antioxidant enzymes, GST and SOD in liver and kidney tissues decreased significantly when rats were subjected to renal I/R. Administration of thymoquinone restored the antioxidant enzymes activity in both organs (Table 2).

Effect of thymoquinone on renal I/R-induced changes in liver and kidney function tests

Liver and kidney function tests were assessed by measuring serum ALT and creatinine level, respectively. In the sham group, the mean levels of ALT and creatinine were 43.57 ± 0.41 IU/ml and 0.43 ± 0.18 mg/dl. After induction of renal I/R, these values increased significantly. Thymoquinone improved liver and kidney functions as reflected by decreased ALT and creatinine levels, comparable with that of the sham group (Table 3).

Effect of thymoquinone on renal I/R-induced alterations in CYP3A1 and spermidine/spermine N-1-acetyl-transferase mRNA expression in hepatic and renal tissues

Renal I/R induced a significant rise in CYP3A1 mRNA expression in both liver and kidney tissue by 8.5- and 31.15-fold, respectively, compared with the sham group. Treatment

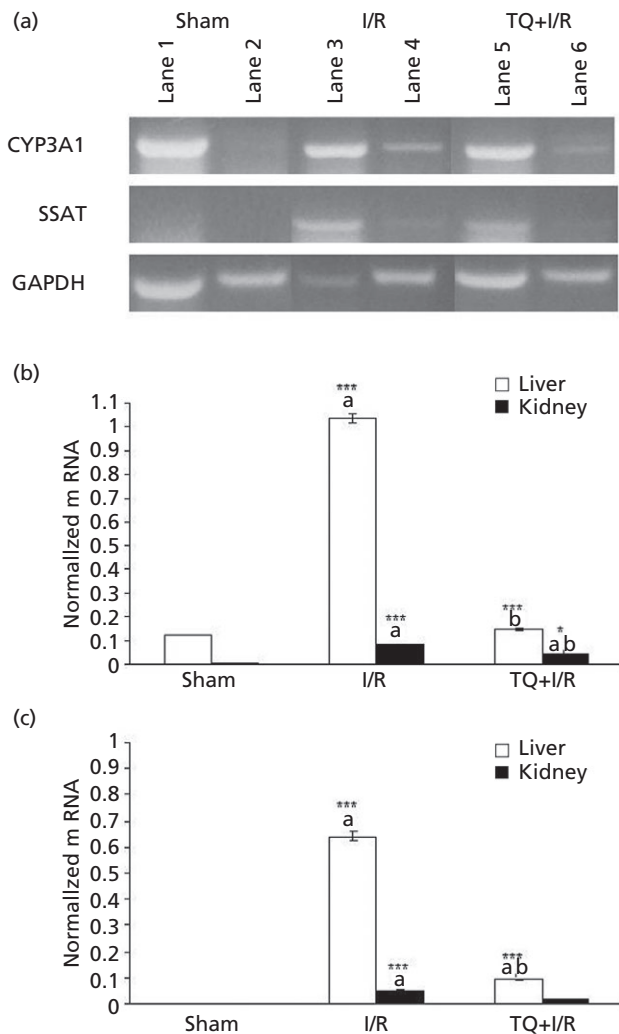


Figure 1 Effect of thymoquinone on CYP3A1 and SSAT mRNA expression in rats liver and kidney tissues after renal ischaemia-reperfusion. Data are represented as the mean \pm SEM ($n = 6$) in each group. (a) Amplified products of CYP3A1, spermidine/spermine N-1-acetyl-transferase (SSAT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes were separated on 1.5% agarose. Lanes 1 and 2: sham-operated group. Lanes 3 and 4: renal ischaemia-reperfusion (I/R) group. Lanes 5 and 6: thymoquinone (TQ) administered orally for 10 days before renal I/R. Lanes 1, 3 and 5 represent liver tissues. Lanes 2, 4 and 6 represent kidney tissues. (b) Expression of CYP3A1 normalized to GAPDH as described under Materials and Methods section. (c) Expression of SSAT normalized to GAPDH as described under Materials and Methods section. ^aSignificantly different from sham group. ^bSignificantly different from renal I/R group. * $P < 0.05$, *** $P < 0.001$.

with thymoquinone decreased this expression by 85.65 and 47.65% in hepatic and renal tissues, respectively (Figure 1a and 1b).

Concerning SSAT mRNA expression, it was not detected in liver and kidney of the sham group. Renal I/R induced a significant rise of the enzyme mRNA expression in both tissues. Thymoquinone decreased significantly SSAT mRNA expression in liver tissue compared with the I/R group. In kidney tissue, thymoquinone was able to reduce

markedly, but not significantly, SSAT mRNA expression (Figure 1a and 1c).

Discussion

Renal injury associated with liver disease is an extensively encountered clinical problem of varied aetiology and high mortality. I/R injury induces an inflammatory response, which results in the formation of ROS that augments local tissue damage or affects organs remote from the site of I/R.^[24]

Kielar *et al.*^[25] evaluated the extrarenal regulation of acute renal ischaemic injury. This regulation may be as a result of increased production of cytokines such as tumour necrosis factor- α and growth factors such as hepatocyte growth factor produced by extrarenal organs.^[26] Renal ischaemia results in increased interleukin (IL)-6 mRNA expression, renal production of IL-6, and expression of IL-10 receptors. IL-6 stimulates the production of IL-10 by the liver, which might ameliorate renal injury.^[27]

The results of our work demonstrated that renal ischaemia for 30 min followed by reperfusion for 4 h led to the following: renal and liver dysfunction (manifested by significant increase of serum creatinine and ALT compared with sham-operated group) (Table 3); significant increase of lipid peroxidation and decrease of antioxidant enzyme activity (GST, SOD) in kidney and liver compared with sham-operated group (Table 2); significant upregulation of CYP3A1 and SSAT gene expression in liver and kidney compared with sham-operated group (Figure 1).

With regards to the effect of I/R on liver and kidney function, many studies have suggested cross-talk between the liver and kidneys.^[1,28] Kadkhodae *et al.*^[28] concluded that a minimum of 45-min ischaemia was needed to study the effects of renal injury on the liver as a remote organ. Rats subjected to 30-min renal ischaemia developed renal impairment without significant hepatic impairment or change in hepatic glutathione.

Those above results did not apparently agree with ours. This may have been due to those authors using only a reperfusion time of 1 h, while reperfusion time used in this study was 4 h. The reperfusion time is an incremental factor in renal I/R injury. Despite the unequivocal benefit of reperfusion of blood to an ischaemic tissue, reperfusion itself can elicit a cascade of adverse reactions that paradoxically injure tissue due to production of reactive oxygen intermediates.^[29]

An ischaemic period usually ranges between 30 and 60 min in renal I/R rat models. Many researchers have used 30-min ischaemia to induce acute renal failure followed by different durations of reperfusion.^[30–32] In mice, 30 min of ischaemia was sufficient to elicit remote effects of I/R injury.^[4]

Concerning the effect of renal I/R on oxidative stress state of renal and hepatic tissue, the results of this study were in agreement with most of the literature. It is generally accepted that ROS and inflammatory leucocytes play an important role in the pathogenesis of renal I/R injury. Renal injury induces an inflammatory response, which results in the formation of ROS that augments local tissue damage or affects organs remote from the site of I/R.^[28]

Acute renal injury may increase, decrease, or have no effect on hepatic drug metabolism.^[33, 34] In this study, we have reported upregulation of CYP3A1 gene expression in the I/R-induced injury group in both kidney and liver. These results were contradictory with some authors. Wang *et al.*^[1] demonstrated that hepatic CYP3A1 and CYP2E1 activity as well as their respective mRNA levels were significantly decreased after renal I/R.

A possible explanation for the varied results from the different models of I/R may be due to differing amount of residual renal and liver function. Another possible mechanism is varying degrees of inflammation and cytokine response in different models of I/R. Indeed, it was reported that renal I/R caused a reduction in renal and liver function and structural alteration in an ischaemia time-dependent manner. Differences in the duration of the ischaemia and reperfusion may cause a difference in the extent of induced inflammation and amount of residual renal function.^[1, 28] It is important to mention that acute renal failure induced by uranyl nitrate in rats is accompanied by upregulation of hepatic CYP3A1 and CYP2E1 enzymes.^[35]

On the other hand, upregulation of SSAT gene expression in liver and kidney after renal I/R in this work was in agreement with previous studies.^[28] It was shown that SSAT overexpression itself generated oxidative stress in cultured kidney cells.^[36] Ryu *et al.*^[6] demonstrated that SSAT was induced in ischaemic rat myocardium and that it was upregulated at the transcriptional level functioning as a cardioprotectant under ATP-depleted conditions. Those authors explained the unexpected results by the possibility that SSAT may have played a helpful role in cell survival under various pathological conditions.

Thymoquinone is the main active ingredient of *N. sativa* seeds, one of the most promising medicinal plants with many therapeutic effects. The most important reported effect of thymoquinone is its antioxidant activity. It acts as an anion scavenger that neutralizes oxygen radicals.^[37] Yildiz *et al.*^[38] investigated the protective effects of *N. sativa* against I/R injury of kidneys. They found that *N. sativa* was effective in reducing serum urea and creatinine levels as well as decreasing the tubular necrosis score. *N. sativa* treatment significantly reduced oxidative stress index and increased total antioxidant capacity levels in both kidney tissue and blood.

In this study, thymoquinone administration led to improvement of both renal and hepatic function in addition to antioxidant enzyme (SOD and GST) activity in both organs. This was in accordance with previous studies proving that thymoquinone was beneficial in restoring declined SOD activity due to I/R insult in rat stomach and forebrain.^[39, 40] Similarly, thymoquinone was able to increase GST activity in different models of oxidative stress-induced pathogenesis such as gastric mucosal injury and diabetes mellitus.^[41, 42]

Thymoquinone was able to afford protection against lipid peroxidation, induced by renal I/R, in kidney and liver. The same effect was obtained when thymoquinone was used to protect against I/R in different organs.^[11, 13, 39, 40]

It was also shown that antioxidant activity of thymoquinone was due to upregulation of antioxidant gene expression.^[43] Anti-inflammatory and anticancer activity of thymoquinone was shown to be related to downregulation of

the expression of cyclooxygenase-2 protein and nuclear factor-kappa B-regulated antiapoptotic gene products, respectively.^[44, 45] Hence, the antioxidant, anti-inflammatory and anticancer properties of thymoquinone continue to be attractive targets that need further investigation to be fully understood.

In this work, we have studied the effect of thymoquinone treatment on changes in CYP3A1 and SSAT mRNA expression induced by I/R. CYP3A1 gene expression was downregulated in liver and kidney compared with the I/R group. SSAT gene expression was downregulated significantly in liver and markedly in kidney. Decrease of SSAT gene expression may lead to reduction of hydrogen peroxide production and hence decrease oxidative stress.

Conclusions

As far as we know this study is the first reported work evaluating the effect of thymoquinone on renal I/R-induced injury on kidney and liver function. The protective effect of thymoquinone was not only due to improvement of antioxidant enzyme activity and decreased lipid peroxidation, we showed that it decreased CYP3A1 and SSAT gene expression. Our results have added further evidence for the beneficial effects of thymoquinone, a molecule that still needs more investigation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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