Original Research

Attenuation of oxidative stress-induced vascular endothelial dysfunction by thymoquinone

Dina S El-Agamy and Manar A Nader

Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt
Corresponding author: Dr Manar A Nader. Email: manarahna@yahoo.com

Abstract

This study aimed to assess the effects of thymoquinone (TQ) on pyrogallol-induced endothelial dysfunction in isolated rabbit aorta. The protective effects of TQ were examined by incubating aortic rings in TQ concomitant with pyrogallol. The results revealed that pyrogallol produced significant enhancement of phenylephrine-induced contraction and impairment of acetylcholine-induced relaxation. Pyrogallol caused a significant increase in lipid peroxidation and reduction in the level of superoxide dismutase and reduced glutathione in the aortic homogenates. In addition, pyrogallol produced a significant decrease in nitrite/nitrate concentrations (NOx), constitutive nitric oxide synthase (cNOS) activity and an increase in inducible nitric oxide synthase (iNOS) activity in the aortic homogenates. These changes were counteracted by TQ co-incubation as TQ attenuated pyrogallol-induced impairment in vascular reactivity in a dose-dependent manner. Furthermore, TQ showed potent antioxidant activity as well as causing a significant increase in NOx and cNOS activity, and depression in iNOS activity. These results suggest that TQ can protect against pyrogallol-induced endothelial dysfunction which probably results from its potent antioxidant capacity that leads to an increase in NO production as well as its ability to enhance the generation and bioavailability of NO.

Keywords: thymoquinone, aorta, oxidative stress, pyrogallol, rabbits

Experimental Biology and Medicine 2012; **237**: 1032–1038. DOI: 10.1258/ebm.2012.012107

Introduction

The endothelium plays a critical role in the regulation of vascular function and the development of physiological and pathophysiological inflammation.[1] Endothelial dysfunction is a state of generalized alteration in endothelial cell phenotype and function characterized by an abnormal vasodilator response, decreased prostacyclin release, increased production of vasoconstrictor substances, impaired endothelial control of fibrinolysis and inflammation, and compromised expression of adhesion molecules. The mechanisms for these endothelial changes are not fully understood, but impaired nitric oxide (NO) bioactivity is a principle feature of this abnormality.[2] NO is generated by the endothelium through the activity of the endothelial nitric oxide synthase (eNOS).[3] In this regard, loss of the ability of the endothelium to release NO results in vascular abnormalities such as vasoconstriction, platelet activation and adhesion of blood elements to the endothelial surface.[2] The decreased availability of NO is also affected by its rate of degradation. NO is degraded by combining with superoxide anion to form peroxynitrite, which is toxic to cells and oxidizes lipids, protein and DNA.[4] Superoxide dismutase (SOD) located between the endothelium and vascular smooth muscle cell scavenges superoxide anion to prevent NO degradation.[5] Emerging evidence suggests that excessive production of reactive oxygen species (ROS) plays a key role in endothelial dysfunction development[6] and hence contributes to cardiovascular diseases including vascular inflammation, endothelial dysfunction, hypertension and heart failure, as ROS diminish the vasodilatory, anti-inflammatory and antithrombotic properties of endothelium.[7,8]

Thymoquinone (TQ; 2-isopropyl-5-methyl-1,4-benzoquinone) is the major component of the volatile oil of *Nigella sativa* seeds. Previous studies have shown the diverse pharmacological actions of TQ such as antibacterial,[9] antihypertensive,[10] antidiabetic,[11] neuroprotective,[12] anti-inflammatory[13] and hypolipidemic.[14] The antioxidant activity of TQ against experimental oxidative injury is well known. Oral administration of TQ protected several organs against oxidative damage induced by free radical-generating agents.[15] TQ possesses strong antioxidant properties through the scavenging ability of different free radicals, its scavenging power being as effective as SOD against superoxide anions.[16,17]

The aim of our study was to examine the possible effects of TQ on endothelial dysfunction induced by pyrogallol in rabbit isolated aortic rings and investigate its possible mechanism(s).

Materials and methods

Experimental animals

Twenty-four male New Zealand White rabbits weighing 1.5 ± 0.2 kg were obtained from the animal center of Faculty of Pharmacy, Mansoura University, Mansoura, Egypt. Rabbits were housed individually in standard stainless steel cages at $24 \pm 2^{\circ}$C with a 12-h light:dark cycle and allowed free access to standard food (El Nasr Lab Chem. Co., Cairo, Egypt) and tap water. All experiments were performed in accordance with the protocol approved by the Committee of Animal Experimentation of Mansoura University.

Chemicals

Pyrogallol, TQ, Tiron, phenylephrine (PE), acetylcholine (ACh) and sodium nitroprusside (SNP) were purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals and biochemicals used in this study were of high analytical grade. TQ was dissolved in 20% ethanol and then diluted several times with saline to the required concentrations before use (final concentration 0.35%). All drugs were added to the organ bath in a final volume of 0.1 mL.

Preparation of rabbit aortic rings

Rabbits were anaesthetized by the administration of ketamine (50 mg/kg) and xylazine (10 mg/kg) into the marginal ear vein. Under anesthesia, the thorax was opened and the descending thoracic aortas were carefully and rapidly removed and freed of adhering soft tissue. The aorta was cut into rings (2–4 mm in length) and immediately placed in a cold oxygenated physiological salt solution (PSS, Krebs–Ringer bicarbonate solution) of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ $7H_2O$ 1.2, $NaHCO_3$ 25 and glucose 11.1, pH 7.4. The aortic rings were divided into four groups each of six preparations (only one segment from one rabbit was used). Aortic rings were mounted between two stainless-steel hooks in an organ bath filled with 18 mL of PSS at a temperature of 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. A passive tension of 2 g was applied and the rings were equilibrated for 60 min with the PSS solution changed every 15 min. Isometric tension generated by the vascular smooth muscle was measured using a force displacement transducer (K30; Hugo Sachs Elektronik, March-Hugstetten, Germany) and recorded with a Powerlab unit linked to a PC running Chart v4.2 software (AD Instruments Ltd, Chalgrove, Oxfordshire, UK). The software was adjusted to record 20 samples per second at 2 mV sensitivity and a 20 Hz filter was applied to remove any noises. After the equilibration period, the rings were exposed to 80 mmol/L KCl for three times to evoke the maximal contraction and then the rings were washed until tension returned to the base line.

Experimental design

Measurement of the contractile function of rabbit aortic rings

Cumulative concentration–response curves (CRCs) were constructed to PE (10^{-9}–10^{-5} mol/L). Contraction responses were expressed as the percentage of the maximum response induced by KCl before. The maximum effect (E_{max}) and concentration inducing 50% of E_{max} (EC_{50}) were determined from the CRC, and pD_2 (negative logarithm to base 10 of the EC_{50} values) was calculated as $-\log EC_{50}$.

Measurement of the relaxant function of rabbit aortic rings

To measure vasorelaxation, aortic rings were first precontracted with PE (10^{-6} mol/L). When the maximal contractile plateau was reached, ACh (10^{-9}–10^{-5} mol/L) or NO-releasing agent SNP was added to the bath cumulatively. Relaxant responses were expressed as the percentage decreases of the magnitude of the contraction induced by PE before the application of ACh or SNP. The maximum effect (E_{max}) and inhibitory concentration 50% (IC_{50}) were determined from the CRC, and pD_2 was calculated as $-\log IC_{50}$.

Effect of TQ on the vasoreactivity of rabbit aortic rings preincubated with pyrogallol

Arterial dysfunction was induced through incubation of the aortic rings with pyrogallol.[18] The aortic rings were randomly subjected to the different treatments as follows:

(1) *Control group*: aortic rings were incubated in TQ vehicle for 30 min;
(2) *Pyrogallol group*: aortic rings were incubated in PSS with 500 μmol/L pyrogallol for 15 min;
(3) *TQ + pyrogallol groups*: aortic rings were preincubated with TQ (10^{-5}, 10^{-4} or 10^{-3} mol/L) for 30 min and 500 μmol/L pyrogallol for the last 15 min;
(4) *Tiron + pyrogallol group*: aortic rings were preincubated with Tiron (a cell permeable superoxide scavenger, 10^{-2} mol/L) and 500 μmol/L pyrogallol for 15 min. This group served as a positive control group.

At the end of the treatments, the rings were incubated in normal PSS without pyrogallol, TQ or Tiron, and then CRCs were constructed to PE to evaluate the contractile function of aorta. To measure vasorelaxation, CRCs were constructed to both ACh or SNP in aortic rings precontracted with PE (10^{-6} mol/L).

Biochemical parameters

The aortic rings were separated and treated according to the same procedure as mentioned before. Aortic rings were collected after the incubation, blotted dry, weighed and then made into a 5% tissue homogenate in ice-cold

0.9% NaCl solution using a variable-speed homogenizer (OMNI International, Kennesaw, GA, USA). Tissue homogenates were centrifuged (1000 *g*, 4°C, 10 min) and supernatants were obtained to measure the following parameters:

Effect of TQ on the oxidative stress induced by pyrogallol

This set of experiments were done to examine the effect of TQ on pyrogallol-induced changes in thiobarbituric reactive substances (TBARs), reduced glutathione (GSH) concentrations and SOD activity in rabbit aortic rings.

Estimation of lipid peroxidation

The TBARs concentration in the homogenate supernatant was measured according to Ohkawa *et al.*[19] Briefly, 1.0 mL of 20% trichloroacetic acid and 1.0 mL of 1% thiobarbituric acid reagent were added to 100 μL supernatant, then mixed and incubated at 100°C for 80 min. After cooling on ice, samples were centrifuged at 1000 *g* for 20 min and the absorbance of the supernatant was read at 532 nm. A standard graph using 1,1,3,3-tetramethoxypropane was plotted to calculate the concentration of TBARs which were expressed as nmol/L/g tissue.

Estimation of SOD activity

The SOD activity was assayed according to the method described by Misra and Fridovich.[20] Briefly, supernatants were added to the reaction mixture (550 μL HCO_3 buffer, 400 μL EDTA, 500 μL epinephrine). Epinephrine undergoes autoxidation rapidly at pH 10.0 to produce adrenochrome, a pink-colored product that was detected spectrophotometrically at 480 nm in the kinetic mode. The amount of enzyme required to produce 50% inhibition was defined as one unit of enzyme activity. The SOD activity was expressed as U/mg protein.

Estimation of reduced GSH

The GSH content was determined according to Sedlak and Lindsay.[21] In brief, an aliquot of the supernatant fraction of the aortic homogenate was mixed with 5% metaphosphoric acid, the mixture centrifuged at 2000 *g* for five minutes, and an aliquot of the clear supernatant was mixed with 1.9 mL of 0.1 mol/L phosphate buffer pH 8.0 and 20 μL of 0.02 mol/L 5,5-dithiobis-2-nitrobenzoic acid in 0.1 mol/L phosphate buffer pH 8.0. The absorbance of the resulting product was measured spectrophotometrically at 412 nm. The concentration of GSH in the sample was derived by reference to a calibration curve of GSH prepared from serial dilutions of a 240 μmol/L GSH stock solution that were treated in an identical manner as the aortic homogenate samples. The results were reported as μmol/L/g tissue.

Effect of TQ on NOx concentrations

The concentration of total NOx was measured in the aortic homogenate as an indicator of NO activity using a NO assay kit according to the supplier's specifications (R&D Systems, Minneapolis, MN, USA). A 0.5% ZnSO4 solution (100 μL) was prepared, then added to supernatant samples (100 μL) and incubated for another five minutes at room temperature. The mixture was centrifuged at 3700 *g* for 20 min at 4°C, and the supernatant was used for measurement of total NOx. The principle of the assay is the conversion of nitrate into nitrite followed by color development with Griess reagent (sulfanilamide and *N*-naphthyl ethylenediamine) in an acidic medium. The absorbance was determined at 540 nm. The amount of NOx in the test samples were determined by interpolation of the result into the standard curve of nitrite prepared from serial dilutions of a 2000μmol/L stock solution. All assays were performed in duplicate and expressed as μM/mg protein.

Determination of NOS activity

Aortic NOS activity was assayed following the steps described by the kit manufacturer (R&D Systems). Briefly, NOS catalyzes L-arginine and O_2 into NO. NO interacts with nucleophilic substances to become a colored compound detected by a spectrophotometer at 530 nm. There are two kinds of NOS, constitutive NOS (eNOS and cNOS) that are activated by Ca^{2+}, and inducible NOS (iNOS) in which activation is Ca^2 independent. To the iNOS assay tube, we added an inhibitor solution which inhibits eNOS and cNOS activity via eliminating Ca^{2+}, and the results represented the iNOS activity.[22,23] For total NOS assay, we did not add the inhibitor solution, and the results represented the total NOS activity. The total NOS activity minus the iNOS activity is the constitutive NOS activity. The NOS activity was expressed as U/mg protein.

Statistical analysis

The results are presented as the mean $\pm$ standard error of mean (SEM), where (*n*) equals the number of animals or preparations. Smooth muscle contraction was calculated as % of maximal steady-state contraction induced by 80 mmol/L KCl. The CRC for each experimental condition was plotted and from it were deduced the values of maximal-agonist induced response (E_{max}) and the concentration of the agonist (expressed as negative log molar concentration) producing 50% of E_{max} (pD_2). The highest response obtained was considered as the maximum response (E_{max}). Non-linear regression analysis (4-parameter curve fit) was carried out using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) and significant differences between groups were determined with one-way analysis of variance followed by Tukey–Kramer multiple comparisons test.

Results

Effect of TQ on the contractile function of isolated rabbit aortic rings preincubated with pyrogallol

Incubation with pyrogallol (500 μmol/L) for 15 min significantly enhanced PE-induced contraction ($P < 0.001$) as compared with the control group. Co-incubation with TQ (10^{-5}, 10^{-4} or 10^{-3} mol/L) or Tiron (10^{-2} mol/L) reduced the enhanced PE-induced contraction as compared with the pyrogallol group (Figure 1).

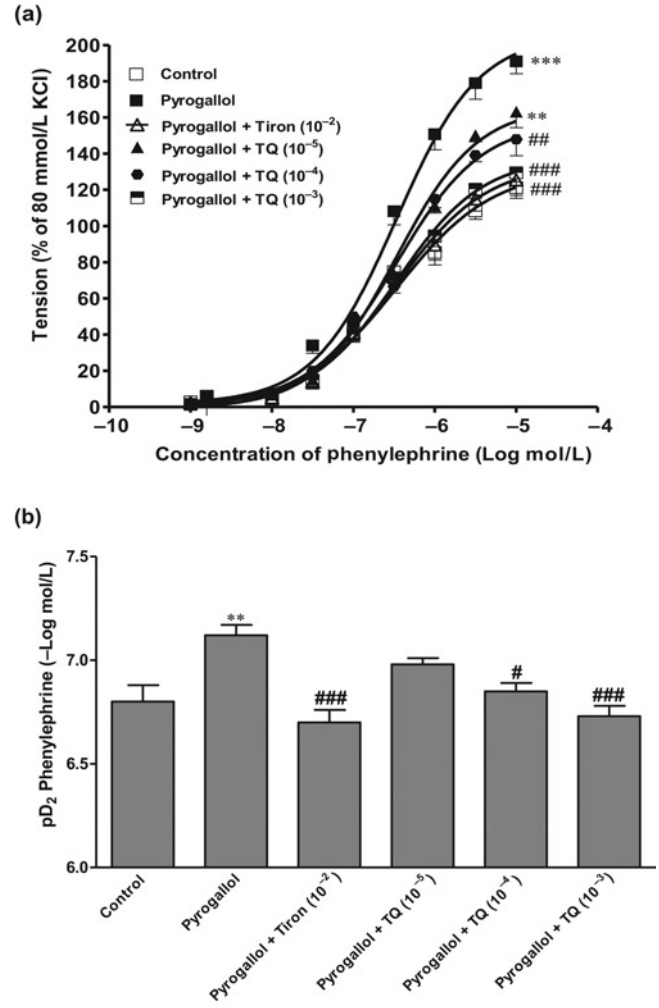

Figure 1 Effect of thymoquinone (TQ; 10^{-5}, 10^{-4} and 10^{-3} mol/L) co-incubation with pyrogallol (500 μmol/L) on phenylephrine (10^{-9}–10^{-5} mol/L)-induced contraction (a) and pD_2 values (b) in aortic rings of rabbits. Tension was measured and calculated as a percentage of the contraction in KCl (80 mmol/L); pD_2 is the negative logarithm of the EC_{50}. Data are expressed as means ± SEM, $n = 6$ rings from six rabbits per group. $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the pyrogallol group (one-way analysis of variance followed by Tukey–Kramer multiple comparisons test)

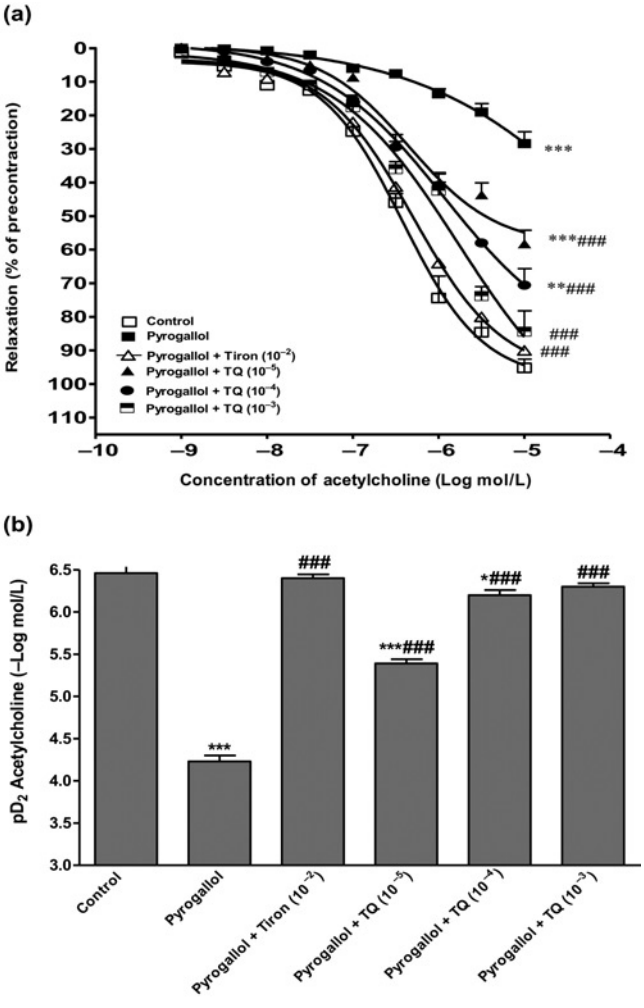

Figure 2 Effect of thymoquinone (TQ; 10^{-5}, 10^{-4} and 10^{-3} mol/L) co-incubation with pyrogallol (500 μmol/L) on acetylcholine (10^{-9}–10^{-5} mol/L)-induced relaxation (a) and pD_2 values (b) in phenylephrine (1 μmol/L)-precontracted aortic rings of rabbits. Tension was measured and calculated as a percentage of the contraction in response to phenylephrine (1 μmol/L); pD_2 is the negative logarithm of the EC_{50}. Data are expressed as means ± SEM, $n = 6$ rings from six rabbits per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the control group. $^{\#\#\#}P < 0.001$ as compared with the pyrogallol group (one-way analysis of variance followed by Tukey–Kramer multiple comparisons test)

Effect of TQ on the relaxant function of isolated rabbit aortic rings preincubated with pyrogallol

Endothelium-dependent and -independent relaxations were produced by ACh and SNP, respectively, in PE (10^{-6} μmol/L) precontracted isolated rabbit aortas in a dose-dependent manner. Co-incubation with pyrogallol (500 μmol/L) for 15 min significantly attenuated ACh-induced endothelium-dependent relaxation as compared with the control group. Treatment with TQ (10^{-5}, 10^{-4} or 10^{-3} mol/L) or Tiron (10^{-2} mol/L) produced a significant reduction ($P < 0.001$) in pyrogallol-provoked attenuation of ACh-induced endothelium-dependent relaxation in a dose-dependent manner (Figure 2). Moreover, there was no difference in the maximal vasorelaxation response to ACh between Tiron- and TQ-treated aortic segments. However, SNP-induced endothelium-independent relaxation did not significantly change among the different experimental groups (data not shown).

Effect on antioxidant status

The lipid peroxidation as assessed in terms of measuring TBARs was significantly increased in the case of incubation with pyrogallol as compared with the control group ($P < 0.001$). However, treatment with TQ (10^{-5}, 10^{-4} or 10^{-3} mol/L) significantly attenuated pyrogallol-induced increase in TBARs in a dose-dependent manner (Table 1). Co-incubation of isolated aortic rings with pyrogallol resulted in a significant reduction in SOD activity ($P < 0.001$) and GSH content ($P < 0.001$) in aortic homogenates as compared with the control group. As depicted in Table 1, TQ or Tiron treatment induced a significant increase in the SOD

Table 1 Effect of thymoquinone (TQ) on pyrogallol-induced change in thiobarbituric acid reactive substances (TBARs), glutathione (GSH) and superoxide dismutase (SOD) in the aortic homogenates of rabbits

Parameter	Control	Pyrogallol	Pyrogallol + Tiron (10^{-2})	Pyrogallol + TQ (10^{-5})	Pyrogallol + TQ (10^{-4})	Pyrogallol + TQ (10^{-3})
TBARS (nmol/g tissue)	18.54 ± 1.6	$35 \pm 2.7^{***}$	$18.5 \pm 1.9^{\#\#\#}$	$27.2 \pm 2.36^{*}$	$21 \pm 1.52^{\#\#\#}$	$18.6 \pm 1.58^{\#\#\#}$
SOD (U/mg protein)	6.1 ± 0.37	$2.5 \pm 0.12^{***}$	$5.7 \pm 0.5^{\#\#\#}$	$3 \pm 0.28^{***}$	$4.5 \pm 0.33^{**\#\#\#}$	$5.3 \pm 0.21^{\#\#\#}$
GSH (μmol/g tissue)	10 ± 0.87	$3.2 \pm 0.42^{***}$	$9.9 \pm 0.9^{\#\#\#}$	$6.2 \pm 0.6^{**\#}$	$7.5 \pm 0.65^{\#\#}$	$9.4 \pm 0.75^{\#\#\#}$

Data are expressed as means $\pm$ SEM, $n = 6$ rings from six rabbits per group
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with the untreated control group
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the pyrogallol group (one-way analysis of variance followed by Tukey–Kramer multiple comparisons test)

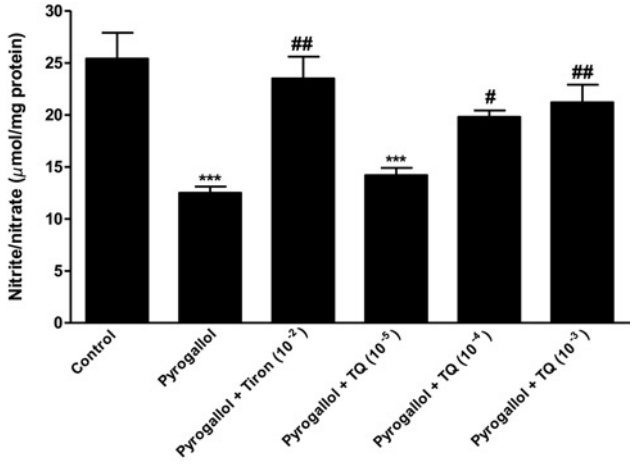

Figure 3 Effect of thymoquinone (TQ; 10^{-5}, 10^{-4} and 10^{-3} mol/L) on pyrogallol (500 μmol/L)-induced change in nitrite/nitrate concentrations in aortic homogenates of rabbits. Data are expressed as means $\pm$ SEM, $n = 6$ rings from six rabbits per group. $^{***}P < 0.001$ as compared with the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ as compared with the pyrogallol group (one-way analysis of variance followed by Tukey–Kramer multiple comparisons test)

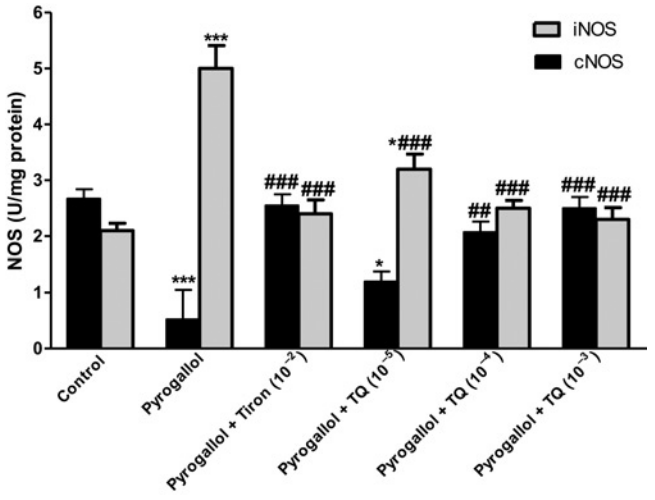

Figure 4 Effect of thymoquinone (TQ; 10^{-5}, 10^{-4} and 10^{-3} mol/L) on pyrogallol (500 μmol/L)-induced change in nitric oxide synthase (NOS) activities in aortic homogenates of rabbits. Data are expressed as means $\pm$ SEM, $n = 6$ rings from six rabbits per group. $^{*}P < 0.05$, $^{***}P < 0.001$ as compared with the control group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared with the pyrogallol group (one-way analysis of variance followed by Tukey–Kramer multiple comparisons test)

activity and GSH content in a dose-dependent manner as compared with the pyrogallol group.

Effect on NOx concentrations, and iNOS and cNOS activities

As shown in Figure 3, pyrogallol (500 μmol/L) caused a significant decrease ($P < 0.001$) in NOx concentrations as compared with the control group. This effect was counteracted by treatment with TQ (10^{-5}, 10^{-4} or 10^{-3} mol/L) which significantly increased the NOx level in a dose-dependent manner. Pyrogallol resulted in a significant increase ($P < 0.001$) in iNOS activity and a decrease ($P < 0.001$) in cNOS activity in aortic homogenates as compared with the control group (Figure 4). Either TQ or Tiron significantly attenuated these changes as they decreased iNOS activity and increased cNOS activity as compared with the pyrogallol group.

Discussion

The present study aimed to test whether TQ has a beneficial effect against pyrogallol-induced endothelial dysfunction in isolated rabbit aorta and to investigate the possible mechanism(s). Furthermore, in this study, Tiron, a potent superoxide scavenger, was used as a positive control to evaluate the antioxidant efficiency of TQ. The results obtained demonstrate the protective effect of TQ against the oxidative damage induced by pyrogallol exposure in rabbit aortic rings. Endothelial dysfunction induced by pyrogallol (500 μmol/L) was depicted by both an increase of PE-induced contraction and the decrease of ACh-induced endothelium-dependent relaxation. On the other hand, pyrogallol did not alter the SNP-induced endothelium-independent relaxation. TQ ameliorated both the enhancement of PE-induced contraction and the impairment of ACh-induced endothelium-dependent relaxation in a dose-dependent manner and this effect may be partially explained through scavenging ROS and increase in NO concentration. Moreover, results have shown that TQ may be as much potent as Tiron in scavenging free radicals.

Endothelial dysfunction plays a key role in the pathogenesis of various cardiovascular disorders such as atherosclerosis, hypertension and coronary artery disease.[6,24,25] The impairment in vascular endothelium integrity, decrease in serum and aortic nitrite/nitrate concentrations and reduction in ACh-induced endothelium-dependent relaxation have been documented to be an index of experimental

endothelial dysfunction.[26,27] The pathogenic relationships among increased oxidative stress, endothelial dysfunction and its cardiovascular complications are well established.[28,29] Previous studies have reported that endothelial dysfunction can be induced by superoxide ion ($O_2^{\bullet -}$) donor, pyrogallol, which rapidly auto-oxidizes in an oxygen-containing aqueous medium to generate $O_2^{\bullet -}$[18] and thus has been used to induce damage from ROS *in vitro*.[30] This effect of pyrogallol has been attributed to the generation of superoxide ion, which then inactivates the NO responsible for ACh relaxation.[31,32]

Results of the present study have shown that exposure of aortic rings to pyrogallol (500 μmol/L) enhance PE-induced contraction and impair ACh-induced relaxation. It has been reported that α1-adrenoceptor-mediated vasoconstriction can be modulated by NO released by the activation of adrenergic receptors on endothelial cell membrane.[33] In this study, it is possible that blockade of this NO release may explain the pyrogallol-induced increase in contractile response to PE. It is also possible that pyrogallol enhancement of PE contraction contributes to its attenuation of ACh-induced relaxation. Previous studies have shown that ACh induces NO release from the endothelium via increasing intracellular calcium in endothelial cells. NO diffuses into adjacent smooth muscle cells and leads to soluble guanylyl cyclase (sGC) activation, cyclic GMP (cGMP) elevation and ultimately to vascular smooth muscle relaxation.[34] The biochemical results of the present study indicated that pyrogallol decreased the aortic concentrations of NOx and cNOS activity and increased iNOS activity that may be responsible for the impairment of ACh-induced relaxation. Putting these findings together, this study suggests that the mechanism by which pyrogallol enhanced PE-induced contraction and inhibited ACh-induced relaxation resides mainly upstream of the Ach–eNOS–NO–sGC–cGMP cascade and NO modulation of α1-adrenoceptor.

$O_2^{\bullet -}$ is a major component of ROS and the direct inactivation of NO by $O_2^{\bullet -}$ is a key event in impairing the endothelium-derived NO bioactivity. The underlying mechanism is probably that NO readily reacts with $O_2^{\bullet -}$ to generate the highly reactive molecule peroxynitrite,[35] and triggers a cascade of harmful events, such as apoptosis, causing uncoupling of NOS, which produces $O_2^{\bullet -}$ instead of NO.[36] All these reactions contribute to the pathogenesis of endothelial dysfunction, including impairment of endothelium-dependent relaxation. Furthermore, pyrogallol impaired the antioxidant status as it significantly decreased the level of the antioxidant enzymes in the aortic homogenate, leading to increased TBAR concentration. These results suggest that pyrogallol-induced endothelial dysfunction is mediated through oxidative stress which is consistent with previous investigations.[37,38]

Results of the present study have shown the beneficial effect of TQ against pyrogallol-induced endothelial dysfunction in a dose-dependent manner. Moreover, *in vitro* co-incubation of aortic rings with Tiron, a superoxide scavenger, significantly improved vascular response to PE and ACh. Treatment with TQ markedly inhibited the enhancement of PE-induced contraction and impairment of ACh-induced endothelium-dependent relaxation in aortic rings exposed to pyrogallol. This beneficial effect of TQ was prominent in the biochemical results as TQ showed potent antioxidant activity. TQ attenuated lipid perioxidation and increased antioxidant enzymes activities. These results are inconsistent with many previous studies that pointed to the effect of TQ as inhibiting tissue inflammation and oxidative stress.[9–13,39] It has been reported to have strong antioxidant potentials through the scavenging ability of different free radicals, its scavenging power being as effective as SOD against superoxide anions.[16,17] It acts as a scavenger of superoxide, hydroxyl radicals and singlet molecular oxygen.[39,40] Furthermore, recent studies have demonstrated that TQ supplementation increases the expression of antioxidant genes, SOD, catalase and glutathione peroxidase, in rat liver. Thus, TQ may reduce oxidative stress through a direct antioxidant effect as well as induction of endogenous antioxidant enzymes.[41] So many previous studies have shown that pretreatment with TQ protected organs against oxidative damage induced by a variety of free radical-generating agents, including cisplatin,[40] carbon tetrachloride[42] and doxorubicin.[43]

Furthermore, NO level and cNOS activity in aortas incubated with pyrogallol were normalized following treatment with TQ. These results are consistent with the previous studies that reported that TQ possesses a potent effect on iNOS activity. TQ inhibited iNOS mRNA expression in rat lipopolysaccharide-stimulated peritoneal macrophage cells,[15,44] which has been attributed to its ability to reduce oxidative stress-induced inflammation leading to the prevention of iNOS upregulation.

Taken together, the overall observed beneficial effect of TQ in preventing vascular endothelial dysfunction may be due to reduction of oxidative stress and increase in NO bioavailability.

Author contribution: DSE-A contributed to the protocol development and the concept of this study, and MAN performed data analysis. Both authors contributed to all other aspects of this study.

REFERENCES

1 Hartge MM, Unger T, Kintscher U. The endothelium and vascular inflammation in diabetes. *Diabetes Vasc Dis Res* 2007;**4**:84–8
2 Voetsch B, Jin RC, Loscalzo J. Nitric oxide insufficiency and atherothrombosis. *Histochem Cell Biol* 2004;**122**:353–67
3 Förstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, Kleinert H. Nitric oxide synthase isozymes: characterization, purification, molecular cloning, and functions. *Hypertension* 1994;**23**:1121–31
4 Heijnen CGM, Haenen GRMM, Wiseman SA, Tijburg LBM, Bast A. The interaction of tea flavonoids with the NO-system: discrimination between good and bad NO. *Food Chem* 2000;**70**:365–70
5 Stralin P, Karlsson K, Johansson BO, Marklund SL. The interstitium of the human arterial wall contains very large amount of extracellular suoeroxide dismutase. *Arterioscler Thromb Vasc Biol* 1995;**15**:2032–6
6 Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel M. Endothelial dysfunction, oxidative stress and risk of cardiovascular events in patients with coronary artery disease. *Circulation* 2001;**104**:2673–8
7 Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. *Arterioscler Thromb Vasc Biol* 2003;**23**:168–75

8 Balakumar P, Koladiya RU, Ramasamy S, Rathinavel A, Singh M. Pharmacological interventions to prevent vascular endothelial dysfunction: future directions. *J Health Sci* 2008;**54**:1–16
9 Hanafy MS, Hatem ME. Studies on the antimicrobial activity of *Nigella sativa* seed (black cumin). *J Ethnopharmacol* 1991;**34**:275–8
10 El-Tahir K, Ashour M, Al-Harbi M. The cardiovascular actions of the volatile oil of the black seed (*Nigella sativa*) in rats: elucidation of the mechanism of action. *Gen Pharmacol* 1993;**24**:1123–31
11 Kanter M. Effects of *Nigella sativa* and its major constituent, thymoquinone on sciatic nerves in experimental diabetic neuropathy. *Neurochem Res* 2008;**33**:87–96
12 Al-Majed AA, Al-Omar FA, Nagi MN. Neuroprotective effects of thymoquinone against transient forebrain ischemia in the rat hippocampus. *Eur J Pharmacol* 2006;**543**:40–7
13 Mutabagani A, El-Mahdy SAM. A study of the antiinflammatory activity of *Nigella sativa* L. and thymoquinone in rats. *Saudi Pharm J* 1997;**5**:110–3
14 Nader MA, El-Agamy DS, Suddek GM. Protective effects of propolis and thymoquinone on development of atherosclerosis in cholesterol-fed rabbits. *Arch Pharm Res* 2010;**33**:637–43
15 Nagi MN, Almakki HA, Sayed-Ahmed MM, Al-Bekairi AM. Thymoquinone supplementation reverses acetaminophen-induced oxidative stress, nitric oxide production and energy decline in mice liver. *Food Chem Toxicol* 2010;**48**:2361–5
16 Badary OA, Taha RA, Gamal el-Din AM, Abdel-Wahab MH. Thymoquinone is a potent superoxide anion scavenger. *Drug Chem Toxicol* 2003;**26**:87–98
17 Fouda AM, Daba MH, Dahab GM, Sharaf El-Din OA. Thymoquinone ameliorates renal oxidative damage and proliferative response induced by mercuric chloride in rats. *Basic Clin Pharmacol Toxicol* 2008;**103**:109–18
18 Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974;**47**:469–74
19 Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;**95**:351–8
20 Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. *J Biol Chem* 1972;**247**:3170–5
21 Sedlak J, Lindsay RH. Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Analyt Biochem* 1968;**25**:192–205
22 Way KJ, Young HM, Reid JJ. Diabetes does not alter the activity and localisation of nitric oxide synthase in the rat anococcygeus muscle. *J Auton Nerv Syst* 1999;**76**:35–44
23 Llorens S, Salazar FJ, Nava E. Assessment of the nitric oxide system in the heart, aorta and kidney of aged Wistar–Kyoto and spontaneously hypertensive rats. *J Hypertens* 2005;**23**:1507–14
24 Giansante C, Fiotti N. Insights into human hypertension: the role of endothelial dysfunction. *J Human Hypertens* 2006;**20**:725–6
25 Yang Z, Ming XF. Recent advances in understanding endothelial dysfunction in atherosclerosis. *Clin Med Res* 2006;**4**:53–6
26 Yang TL, Chen MF, Luo BL, Yu J, Jiang JL, Li YJ. Effect of fenofibrate on LDL-induced endothelial dysfunction in rats. *Naunyn Schmiedebergs Arch Pharmacol* 2004;**370**:79–83
27 Balakumar P, Sharma R, Singh M. Benfotiamine attenuates nicotine and uric acid-induced vascular endothelial dysfunction in rats. *Pharmacol Res* 2008;**58**:356–63
28 Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000;**87**:840–4
29 Thomas SR, Chen K, Keaney JF Jr. Oxidative stress and endothelial nitric oxide bioactivity. *Antioxid Redox Signal* 2003;**5**:181–94
30 Bell DR, Gochenaur KE, Hecht J. $O_2(-)$-mediated impairment of coronary arterial relaxation is prevented by overnight treatment with 1 nM beta-estradiol. *J Appl Physiol* 2002;**93**:1952–8
31 Moncada S, Palmer RMJ, Gryglewski RJ. Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc Natl Acad Sci USA* 1986;**83**:9164–8
32 Carrasco OF, Vidrio H. Endothelium protectant and contractile effects of the antivaricose principle escin in rat aorta. *Vasc Pharmacol* 2007;**47**:68–73
33 Godfraind T, Egleme C, Osachie AI. Role of endothelium in the contractile response of rat aorta to alpha-adrenergic agonists. *Clin Sci* 1985;**68**:65–71
34 Hansen K, Nedergaard OA. Methodological aspects of acetylcholine evoked relaxation of rabbit aorta. *J Pharmacol Toxicol* 1999;**41**:153–9
35 Kissner R, Nauser T, Bugnon P, Lye PG, Koppenol WH. Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem Res Toxicol* 1997;**10**:1285–92
36 Maritim AC, Sanders RA, Watkins JB 3rd. Diabetes, oxidative stress, and antioxidants: a review. *J Biochem Mol Toxicol* 2003;**17**:24–38
37 Machha A, Achike FI, Mohd MA, Mustafa MR. Baicalein impairs vascular tone in normal rat aortas: role of superoxide anions. *Eur J Pharmacol* 2007;**565**:144–50
38 Jin BH, Qian LB, Chen S, Li J, Wang HP, Bruce IC, Lin J, Xia Q. Apigenin protects endothelium-dependent relaxation of rat aorta against oxidative stress. *Eur J Pharmacol* 2009;**616**:200–5
39 Mansour MA, Nagi MN, El-Khatib AS, Al-Bekairi AM. Effects of thymoquinone on antioxidant enzyme activities, lipid peroxidation and DT-diaphorase in different tissues of mice: a possible mechanism of action. *Cell Biochem Funct* 2002;**20**:143–51
40 Badary OA, Nagi MN, Al-Shabanah OA, Al-Sawaf HA, Al-Sohaibani MO, Al-Bekairi AM. Thymoquinone ameliorates the nephrotoxicity induced by cisplatin in rodents and potentiates its antitumor activity. *Can J Physiol Pharmacol* 1997;**75**:1356–61
41 Ismail M, Al-naqeep G, Chan KW. *Nigella sativa* thymoquinone-rich fraction greatly improves plasma antioxidant capacity and expression of antioxidant genes in hypercholesterolemic rats. *Free Radic Biol Med* 2009;**48**:664–72
42 Nagi MN, Alam K, Badary OA, Al-Shabanah OA, Al-Sawaf HA, Al-Bekairi AM. Thymoquinone protects against carbon tetrachloride hepatotoxicity in mice via an antioxidant mechanism. *Biochem Mol Biol Int* 1999;**47**:153–9
43 Al-Shabanah OA, Badary OA, Nagi MN, Al-Gharably NM, Al-Rikabi AC, Al-Bekairi AM. Thymoquinone protects against doxorubicin-induced cardiotoxicity without compromising its antitumor activity. *J Exp Clin Cancer Res* 1998;**17**:193–8
44 El-Mahmoudy A, Matsuyama H, Borgan MA, Shimizu Y, El-Sayed MG, Minamoto N, Takewaki T. Thymoquinone suppresses expression of inducible nitric oxide synthase in rat macrophages. *Int Immunopharmacol* 2002;**2**:1603–11

(Received March 21, 2012, Accepted May 31, 2012)