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Thymoquinone suppresses expression of inducible nitric oxide synthase in rat macrophages

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Abstract

The objective of the present study was to determine the immunomodulatory role of thymoquinone (TQ) regarding its effect on the production of nitric oxide (NO) by rat peritoneal macrophages. Under certain conditions, macrophages and certain other cells can produce high concentrations of NO from its precursor L-arginine via inducible nitric oxide synthase (iNOS) pathway. TQ has been established as the major component of the oil extracted from *Nigella sativa* plant seeds, which is being used frequently in herbal medicine. TQ (IC_{50} 1.4–2.76 μM) dose- and time-dependently reduced nitrite production, a parameter for NO synthesis, in supernatants of lipopolysaccharide (LPS)-stimulated (5 μg/ml) macrophages without affecting the cell viability. The protein level of iNOS in peritoneal macrophages was also decreased by TQ in a concentration-dependent manner. In addition, TQ inhibited the increase in iNOS mRNA expression induced by LPS indicated by reverse transcription-polymerase chain reaction (RT-PCR). These inhibitory effects of TQ were confirmed by immunofluorescence staining of iNOS in macrophages which showed decreased immunoreactivity for iNOS after treatment with TQ if compared with the control LPS-stimulated cells. These results suggest that TQ suppresses the production of NO by macrophages; an effect which may be useful in ameliorating the inflammatory and autoimmune conditions.
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Keywords: Inducible nitric oxide synthase; Macrophage; Nitric oxide; Thymoquinone

1. Introduction

A heavy responsibility is placed on macrophage cells as a shield against various infectious agents because of its phagocytotic, digestive and secretory functions. Through its secretions and receptors, macrophage participates in complex interactions involving cellular and humoral components of the inflammatory and immunologic networks [1].

Among the important products of macrophage is nitric oxide (NO) which is a diatomic free radical molecule that has various biological effects. It is produced from its precursor L-arginine by macrophage

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and other cells via nitric oxide synthase (NOS) pathways. Molecular cloning and sequencing analyses revealed that NO is produced in physiological and pathophysiological conditions by three main types of NOS isoforms: neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II) and endothelial NOS (eNOS or NOS III) [2,3]. While eNOS and nNOS are constitutively expressed and regulated by Ca^{++}/calmodulin, the activity of iNOS is regulated at the transcriptional level by mediators such as interleukins, interferons, inflammatory stimuli and endotoxins including bacterial lipopolysaccharide (LPS). The most prominent physiological actions of NO as a biological mediator include cGMP-dependent vasodilatation, neurotransmission and cytotoxicity against pathogens in the non-specific immune defense mechanism [4]. However, NO appears to be a two-edged sword where evidence indicates that macrophage derived NO is a contributing cause to the development of autoimmune diseases [5–8].

Thymoquinone (TQ) (Fig. 1) was isolated as the principal active ingredient from the volatile oil of *Nigella sativa* seeds [9]. The seeds of the plant were shown to contain a fixed oil (>30% wt/wt) and a volatile oil (0.40–0.45%) [10]. The volatile oil has been shown to contain 18.4–24.0% TQ (2-isopropyl-5-methyl-1,4-benzoquinone) [11]. TQ has been shown to attenuate eicosanoid generation [12], lipid peroxidation [13], cisplatin nephrotoxicity [14], ifosfamide Fanconi syndrome [15], tetrachloride hepatotoxicity [16], doxorubicin cardiotoxicity [17] and histamine release [18].

In spite of these studies, there is no available information on the effect of TQ on the macrophage function regarding its NO secretory potential which is responsible for cytotoxic and injurious consequences if NO is produced in large quantities. Therefore, the aim of the present study is to investigate the possible inhibitory effect of TQ on NO production and iNOS expression in rat peritoneal macrophages; an effect which may play an important modulatory role on macrophage function with consequent protection of host cells from cytotoxicity caused by excess NO.

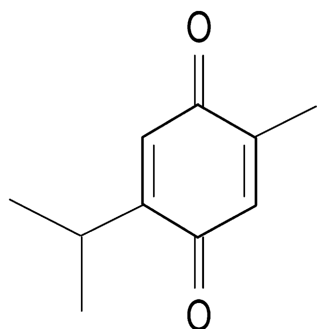

Fig. 1. Structure formula of thymoquinone.

2. Materials and methods

2.1. Preparation of rat peritoneal macrophages

Male Long–Evans Tokushima Otsuka (LETO) rats, specific pathogen-free, and weighing 300–350 g were used. The experimental procedures were approved by Gifu University, Animal Care and Use Committee and were in accordance with Japanese Department of Agriculture guidelines. The method described by Handel-Fernandez and Lopez [19] is adopted for collecting peritoneal macrophages. Briefly, rats were sacrificed by cervical dislocation, placed in such a manner that the abdomen is facing up and completely wet with 70% ethanol. A transverse cut was made in the inguinal area and the skin was dissected to expose the abdominal wall which is then soaked with 70% ethanol. About 20 ml of cold DMEM (Sigma, St. Louis, MO, USA) containing 5% heat-inactivated endotoxin-free fetal bovine serum (FBS) (Sigma) was injected. The needle was removed and the abdomen was gently massaged. The medium was drawn back and the peritoneal fluid was dispensed into 50 ml polypropylene tubes. This procedure was repeated two more times. The cells were washed three times by centrifugation at $300 \times g$ for 10 min at 4 °C and re-suspending them in DMEM medium containing 5% FBS.

2.2. Macrophage culture

The peritoneal cells were suspended in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma) at a density of 5×10^5 cells/ml of the medium. The cell suspension was poured into 6-well tissue culture plates with 2 ml in each well, and the plates were incubated for 2 h at 37 °C and 5% CO_2 tension. The cells were then washed three times

with the medium to remove non-adherent cells. The adherent cells (97% macrophages) were incubated for 20 h at 37 °C and 5% CO_2 tension in medium containing 10% (v/v) FBS for growth. The cells were then washed three times with medium containing no FBS and incubated with the indicated agents.

2.3. Incubation of macrophages with reagents

After three washes with FBS-free medium, the cells were incubated with LPS (5 μg/ml; Sigma) with or without TQ (Aldrich, Milwaukee, WI, USA; purity 99%) for 6, 12 and 24 h at concentrations of 0.5, 1, 10 and 100 μM. Some wells were left without any drugs as negative control while the wells that were incubated only with LPS were the positive control. After the indicated times, the supernatants were collected for measurement of nitrite concentration, while the cells were used for blotting, mRNA expression and staining.

2.4. Viability assay

The viability of the cells was examined by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) [20], which is based on the ability of mitochondrial enzyme, succinate dehydrogenase to cleave MTT to the blue compound formazan. The cells were incubated for the periods indicated in 2 ml of medium containing agents. Then 400 μl of MTT solution in phosphate buffered saline (PBS) (5 mg/ml, pH 7.4) was added to each well and the cells were further incubated for 4 h at 37 °C and 5% CO_2 tension. Then 1 ml of 0.04 N HCl solution in isopropanol was added and the cells were sonicated at 10% maximum power for 3 s and the resultant colored product was read on a Microplate Reader (MPR.A4i II, Tosoh, Tokyo, Japan) at 570 nm. Treatment with drugs at the described concentrations showed no significant changes in cell viability.

2.5. Measurement of nitrite concentration

NO production in culture supernatants was assessed by measuring nitrite, its stable degradation product using Griess reaction as previously described [21]. The medium supernatants were centrifuged and 100 μl from each sample was mixed with an equal amount of Griess reagent in 96-well plates and then incubated for 10 min at room temperature in the dark. Sodium nitrite (Sigma) was used as a standard and absorbance is measured at 540 nm by a Microplate Reader.

2.6. Measurement of iNOS enzyme synthesis by Western blotting analysis

Cellular level of macrophage iNOS protein was estimated by immunoblotting. After removal of the supernatant, the cells were washed with PBS three times, then scrapped off, centrifuged ($1000 \times g$, 5 min, 4 °C) and the pellets were lysed with lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS). The protein concentration of lysates was determined using the Bradford protein assay [22]. Equal amounts of proteins from corresponding samples were used for Western blotting analysis, where they were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% running gels with the Laemmli buffer system [23]. Proteins were transferred to 0.45 μm nitrocellulose membranes and blots were blocked by gentle shaking with 5% non-fat dried milk solution for 1 h. Blots were rinsed twice for 5 min with PBS containing 0.05% Tween-20 and incubated with anti-iNOS polyclonal antibody (1:2000 dilution, Sigma) for 1 h at 37 °C. Membranes then washed extensively five times (5 min each) with PBS containing Tween-20 and then further incubated with horseradish peroxidase-conjugated polyclonal anti-rabbit IgG antibody (1:2000 dilution, Amersham, UK) for 45 min at 37 °C. Again, blots were washed five times (5 min each) with PBS containing 0.05% Tween-20 and subjected for 1 min to a reagent mixture (Amersham) as a substrate indicator for peroxidase. Immunoreactive bands were detected by exposure for at least 3 min to enhanced chemiluminescence (ECL) detection system and developed with Hyperfilm-ECL according to the procedure recommended by the maker (Amersham). Quantification of iNOS and α-tubulin proteins was carried out by densitometric analysis using Scicon Image beta 4.02 (Scicon, Maryland, USA).

2.7. Isolation of RNA and assessment of iNOS mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from rat peritoneal macrophages by guanidium isothiocyanate

method [24,25] using MagExtractor RNA purification kit (Toyobo, Osaka, Japan). iNOS mRNA was assessed by reverse transcription (RT) and polymerase chain reaction (PCR). RT was performed with random primers using enhanced avian HS RT-PCR kit (Sigma). RT reaction reagents were added for each 200 μl thin-walled PCR microcentrifuge tube in ice as follows: 4 μl PCR water, 5 μl of total RNA (50 pg/μl) and 1 μl anchored oligo $(dt)_{23}$. The reagents were mixed gently and the tubes were placed in thermal cycler (Takara, Japan) at 70 °C for 10 min to denaturize RNA secondary structures if any. The tubes were then removed, placed in ice and the following reagents were further added to each of them: 5 μl PCR water, 2 μl 10× buffer for AMV RT (1×), 1 μl deoxynucleotide mix (500 μM dNTP), 1 μl RNase inhibitor (1 U/μl) and 1 μl enhanced avian RT (1 U/μl). First-strand cDNA synthesis was performed by placing the reaction tubes (now each containing 20 μl total volume) in thermal cycler at 25 °C for 15 min and then at 44 °C for 50 min. Afterwards, the tubes were incubated at 99 °C for 5 min to stop the reaction and then kept at 4 °C until PCR was performed.

PCR was performed using the same kit with rat iNOS primers prepared by nucleic acid synthesis system (Rikaken, Nagoya, Japan). The sense and antisense sequences were 5′-GTGTTCCACCAGGAGATGTTG-3′ and 5′-CTCCTGCCCACTGAGTTCGTC-3′, respectively. The cDNA amplification product was proposed to be a 576 bp fragment. To perform PCR, 5 μl of the RT reaction was added to the following reagents in each of the new 200 μl thin-walled PCR microcentrifuge tubes: 5 μl 10× Accu Taq buffer (1×), 1 μl deoxynucleotide mix (200 μM each dNTP), 1 μl control primers (0.4 μM each), 1 μl JumpStart Accu Taq LA DNA polymerase mix and the final volume of the reaction (50 μl) was completed with PCR water. The contents of the tubes were gently mixed and spinned, and then the tubes were placed in a thermal cycler. A first incubation at 95 °C for 2 min was performed for initial denaturation. This was followed by 30 cycles of the following sequential steps: 30 s at 94 °C (denaturation), 1 min at 54 °C (annealing), and 1 min at 72 °C (extension); the final incubation was at 75 °C for 5 min (final extension). The PCR products were size-fractioned by 1.5 agarose gel electrophoresis. After staining with ethidium bromide, DNA bands were visualized with an ultraviolet transilluminator (Toyobo). A negative control of the PCR reaction was included in each set of experiments. Densitometric analysis for iNOS and β-actin was made by the same way as in case of Western blotting.

2.8. Immunostaining of iNOS

Cells grown on tissue culture slides and incubated with the indicated agents in the same way as described above were washed rapidly with PBS and fixed at room temperature for 10 min with 2% paraformaldehyde. After washing, the cells were blocked for 15 min with 1% bovine serum albumin in TTBS (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20) containing 1% Triton X-100. After blocking, the cells were incubated with anti-iNOS antibody (1:2000) for 1 h at 37 °C in humid conditions, then extensively washed with PBS and stained for 45 min with FTIC-labeled anti-rabbit IgG (1:2000). After additional washes with PBS, the walls of the tissue culture slides were removed and the immunostained cells on the slides were observed and photographed using inverted fluorescent microscope (Olympus, Japan).

2.9. Statistical analysis

Data were expressed as means ± S.E. Statistical analysis was performed by Student's *t*-test to express the difference between two groups. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) followed by Dunnett's test. Results in which the p value is < 0.05 were considered significantly different. Routinely, experiments were repeated at least three times with similar or identical results.

3. Results

3.1. Thymoquinone inhibits nitrite production in LPS-activated rat peritoneal macrophages

Resident peritoneal macrophages were cultured in DMEM without serum in the presence of different concentrations of LPS. The concentration of nitrite, as indicator for NO, was measured in cultured supernatants after 24 h. It is evident from Fig. 2 that LPS-

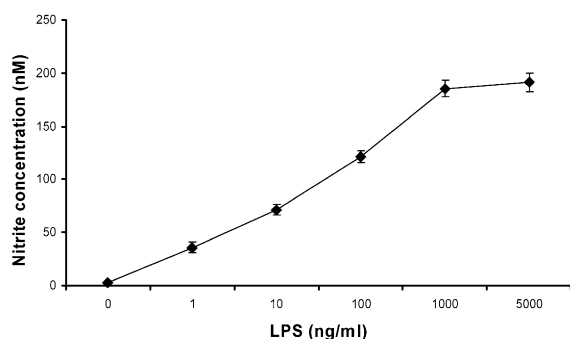

Fig. 2. Dose-dependent effects of LPS on production of nitrite by rat peritoneal macrophages. Macrophages (5×10^5 cells/ml) were incubated for 24 h with various concentrations of LPS. Each value is the mean ± S.E. of three experiments.

induced production of nitrite was concentration-dependent with maximal induction at 5 μg/ml of LPS; thus, it was used all over the following experiments.

As shown in Fig. 3, nitrite production by macrophages was dependent on the activating status of the cells. Unstimulated macrophages, even after 24 h of culture, basally produced negligible levels of nitrite. TQ alone did not significantly affect basal nitrite production. However, stimulating the cells with LPS induced the production of nitrite in dose- and time-dependent manners. TQ (0.5–100 μM) showed a concentration-dependent inhibition of nitrite accumulation by peritoneal macrophages stimulated with LPS. IC_{50} value for TQ after 24 h incubation was 1.4 ± 0.60 μM. DMSO 10% (the vehicle of TQ) had no effect either on cell viability or nitrite production levels. Significant inhibition by TQ was observed at 1 μM and greater than 60% inhibition was noted at concentrations ≥ 10 μM. To exclude the possibility that the inhibitory effect of TQ is due to potential cytotoxicity, the cell viability was assessed by mitochondrial-dependent reduction of MTT to formazan. Cell viability after addition of LPS and/or TQ was still greater than 90% when compared with control. This indicates that the inhibition of nitrite production by TQ was not due to cell death.

We also determined the time course of changes in released nitrite at 6, 12 and 24 h. Upon stimulation of macrophage cells with 5 μg/ml LPS, nitrite was increasingly released from 6 to 24 h as indicated by Griess reaction. TQ suppressed that release in a time-dependent manner. Fig. 3 shows dose- and time-dependent nitrite release by stimulated macrophage and the dose- and time-dependent suppression of that release by TQ. IC_{50} of TQ at 6 and 12 h were 2.76 ± 0.57 and 1.29 ± 0.49 μM, respectively.

3.2. Thymoquinone diminishes iNOS protein levels in LPS-activated rat peritoneal macrophages

The marked inhibition of nitrite production observed in TQ-treated cells suggests that TQ might inhibit iNOS protein synthesis. To determine whether the NO inhibitory effect of TQ is due to inhibition of iNOS protein expression, Western blotting analysis was carried out using a polyclonal antibody for rat macrophage iNOS. Rat peritoneal macrophages were incubated with LPS (5 μg/ml) in the absence and presence of TQ (0.5–100 μM) for 12 h and the cell lysates were examined for the 130 kDa iNOS protein. The dose responses for inhibition of iNOS proteins by TQ are shown in Fig. 4. Obvious inhibition was observed at 1 μM TQ, while 100 μM almost completely abolished iNOS expression. Cell viability, however, seems to be intact in the presence of TQ as α-tubulin protein is unaffected.

The time course changes in synthesized iNOS protein expression were also determined at 6, 12 and 24 h. Upon stimulation of macrophages with LPS, iNOS protein was induced in increasing amounts from

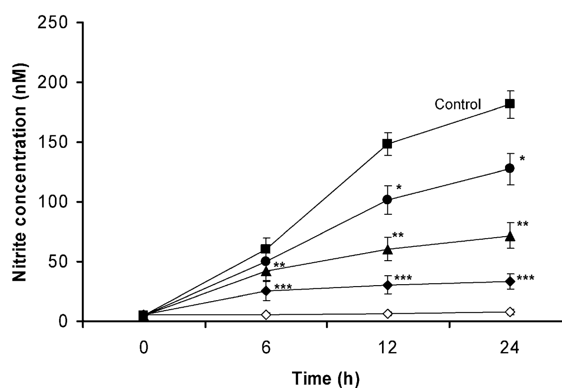

Fig. 3. Time-dependent production of nitrite by rat peritoneal macrophages (5×10^5 cells/ml) incubated for 6, 12 or 24 h without LPS (◇) and with LPS in the absence (■) or presence of thymoquinone 1 μM (●), 10 μM (▲), and 100 μM (◆). Results are representative for three experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

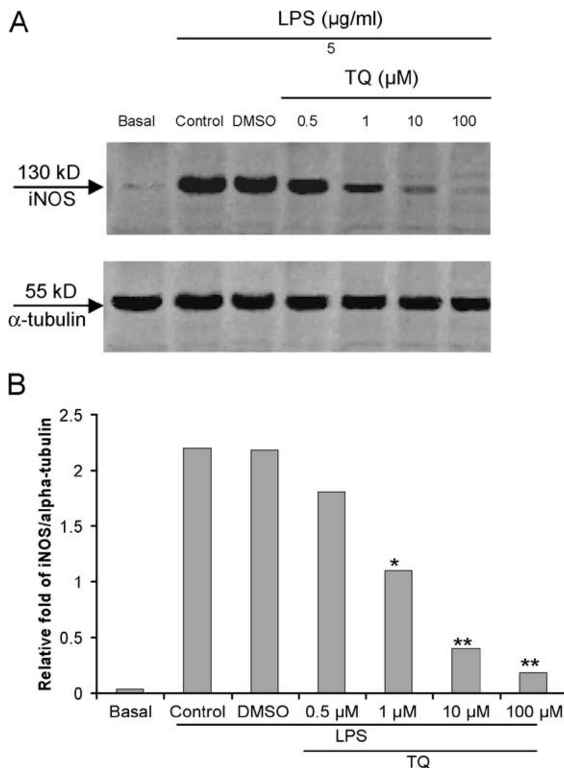

Fig. 4. Concentration-dependent expression of iNOS in LPS-activated rat peritoneal macrophages. (A) Macrophages (5×10^5 cells/ml) were plated in 6-well plates and stimulated with LPS (5 μg/ml) in the absence or presence of TQ (0.5–100 μM) for 12 h. Cells were lysed and its protein was subjected to electrophoresis using SDS-PAGE. The blots were transferred to cellulose membranes which were incubated with rabbit anti-rat iNOS antibody for 1 h then with anti-rabbit IgG for 30 min. The reactions were read by image reader. (B) Band intensities were quantified by densitometer. A representative blot of three experiments with similar results is shown. * $P<0.05$, ** $P<0.01$.

6 to 24 h. TQ (10 μM), in coexistence with LPS, markedly blocked this induction. This result is parallel to the reduction of nitrite production in TQ treated cells. The expression of α-tubulin was normal all over the time courses, suggesting that inhibition by TQ was not due to non-specific or cytotoxic effects.

3.3. Thymoquinone reduces iNOS mRNA expression levels in LPS-activated rat peritoneal macrophages

Since reduced protein levels could be a consequence of reduced corresponding mRNA expression, iNOS mRNA level was examined by RT-PCR. The size of PCR products was estimated by comparison with a size marker and found to correspond to 576 bp for rat macrophage iNOS. The levels of transcripts of iNOS gene in LPS-activated macrophages in the absence or the presence of TQ (0.5–100 μM) were shown in Fig. 5. iNOS gene transcription was reduced by TQ in a concentration-dependent fashion and almost completely blocked by 100 μM of TQ after 12 h. DMSO alone (10%) did not affect the iNOS mRNA expression and there was always a strong and homogenous transcription of the housekeeping gene β-actin, indicating a constant level of cDNA in all samples.

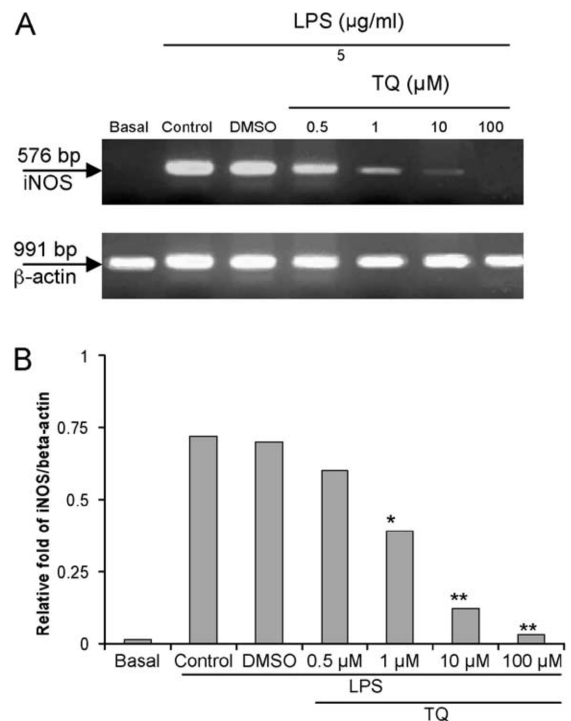

Fig. 5. RT-PCR analysis of iNOS mRNA expression in rat peritoneal macrophages. (A) Macrophages (5×10^5 cells/ml) were incubated with LPS (5 μg/ml) for 12 h or in coexistence of vehicle (DMSO) and various concentrations of TQ (0.5–100 μM). TQ produced dose-dependent inhibition of LPS-induced iNOS mRNA expression. (B) Band intensities were quantified by densitometer. The experiment was repeated three times with similar results. * $P<0.05$, ** $P<0.01$.

3.4. TQ reduced immunoreactivity for iNOS in LPS-activated macrophages

The immunoreactivity for iNOS in cell cultures of rat peritoneal macrophages in the absence or the presence of TQ is shown in Fig. 6. Macrophages treated only with LPS displayed a significant strong reactivity to iNOS antibody which was not detected in untreated cells. While cells treated with LPS + TQ exhibited significant reduction in both strength and amount of iNOS immunoreactivity. Cells were morphologically determined as macrophages following Harris's hematoxylin staining.

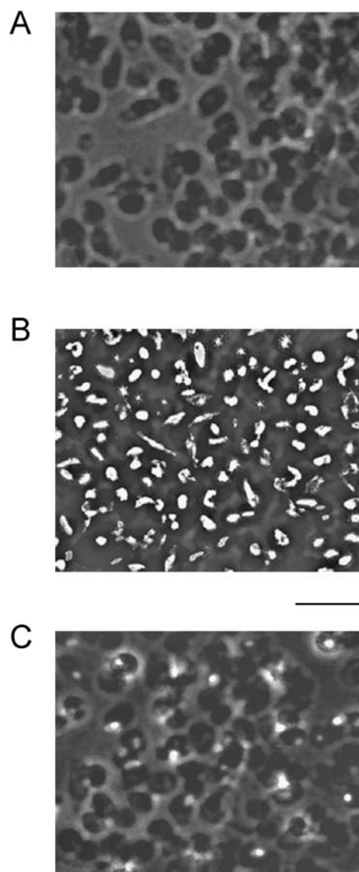

Fig. 6. Immunoreactivity for iNOS protein in rat peritoneal macrophages. Cells were stimulated with LPS (5 μg/ml) in the absence or presence of thymoquinone (10 μM) for 12 h. Adhered cells were washed, fixed and incubated with polyclonal anti-iNOS antibody (1:2000) for 1 h, washed extensively, and stained for 30 min with FTIC-labeled anti-rabbit IgG (1:2000). (A) Control; (B) after treatment with LPS (5 μg/ml); (C) after treatment with LPS + TQ (10 μM). The bar represents 200 μm.

4. Discussion

In the present study, we report that TQ, the active principle isolated from *N. sativa* seeds [9], concentration- and time-dependently suppresses LPS-stimulated NO production by rat peritoneal macrophages. The inhibition was maximal at 100 μM TQ and lasts up to 24 h with IC_{50} about 1.4 μM. Although there is no available information regarding the effect of TQ on excessive NO production by macrophages, yet our results may be parallel to previous reports that TQ has inhibitory effects on some inflammatory mediators. For instance, Houghton et al. [12] reported that pure TQ inhibits cyclooxygenase and 5-lipoxygenase pathways of arachidonate metabolism in rat peritoneal leukocytes with consequent inhibition of the formation of thromboxane B_2 and leucotriene B_4. Chakravarty [18] demonstrated the inhibition of histamine release from mast cells by nigellone, the polymer of TQ. In addition, TQ has been found to protect kidney from cisplatin nephrotoxicity [14], liver from tetrachloride hepatotoxicity [16], and heart from doxorubicin cardiotoxicity [17]. Taken together, previous and present data indicate that TQ may be very beneficial in the treatment of inflammatory and cytotoxic conditions as it potently inhibits a wide spectrum of inflammatory and cytotoxic mediators.

NO production by macrophages is regulated by various factors (LPS, cytokines, 5,6 dimethylxanthenone-4-acetic acid, picolinic acid, cAMP elevating agents, UV light, ozone, trauma and microbes), and the induction of iNOS requires gene transcription and new protein synthesis, since actinomycin-D and cycloheximide inhibited LPS- or interferon-γ-induced NO generation [26–28]. Thus, NO production by iNOS may be regulated at many sites, including transcription, post-transcription, translation, and post-translational modifications [3].

The results shown in the present study reveal that TQ mediates its inhibitory effect on NO production via reduction of iNOS mRNA and protein expressions. In spite of these observations, which may suggest that the NO's inhibitory properties of TQ may involve the regulation of iNOS gene, yet the

precise site of action and whether TQ impairs specific factors involved in the transcriptional machinery or decreases mRNA or iNOS stabilities remain to be investigated. However, results obtained from time course analysis revealed that the inhibition of nitrite synthesis by TQ occurs in a fashion similar to a protein synthetic inhibitor cycloheximide as the inhibitory action of TQ lasts up to 24 h. Unlike the only transcription inhibitor, actinomycin-D, whose effect abolishes after about 6 h from incubation. Therefore, it could be speculated that the mechanism of action of TQ not only involves the iNOS gene, but also extends to affect the synthesis and/or stability of iNOS protein.

Accumulating evidences indicate that endogenous NO contributes to the physiology of almost every organ system, for example, it mediates aspects of macrophage cytotoxicity, regulates blood pressure and participates in neurotransmission [29,30]. However, enhanced formation of NO enlarges its sphere of action, thus it contributes to destruction of the hosts' joints [5,7] and kidneys [5] along the course of local (e.g., polyarthritis, osteoarthritis, nephritis) or systemic inflammatory disorders, diabetes, arteriosclerosis and autoimmune diseases [31–33]. Moreover, it has been found that there is a positive correlation between NOS and tumor progression. Some in vivo experiments showed that in the presence of long-term infusion of a highly selective iNOS inhibitor, rapidly growing invasive tumors have been turned to slower growing non-invasive ones [34]. Thus, it has been established that inhibition of overproduction of NO may have therapeutic benefits in patients with the abovementioned conditions. The data shown in the present study may confer TQ such novel NO inhibitory properties at the molecular level, new evidence that supports the wide traditional use of *N. sativa* in folk medicine for ameliorating and treatment of chronic conditions. TQ, as the major component of the *N. sativa* seed extract, can be used in turn as a cytoprotective remedy against autoimmune and other inflammatory conditions or complications. However, further studies are required to clarify whether our observations have any clinical relevance particularly in chronic conditions where the dominant inflammatory cells are macrophages and inhibition of overproduction of NO at the sites of inflammation can improve the response.

In conclusion, this study demonstrated that TQ (i) reduces nitrite accumulation by LPS-activated macrophages in dose- and time-dependent manners; (ii) markedly reduces iNOS protein expression indicated by Western blotting analysis; (iii) markedly inhibits iNOS gene transcripts if compared to LPS-activated control cells indicated by RT-PCR analysis of iNOS mRNA; (iv) reduces fluorescent immunoreactivity of iNOS protein in macrophages if compared with the strong reactivity of LPS-activated control cells. Such properties may suggest TQ as a potent anti-inflammatory and cytoprotective agent.

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