Original Article

Thymoquinone triggers anti-apoptotic signaling targeting death ligand and apoptotic regulators in a model of hepatic ischemia reperfusion injury

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ABSTRACT: Thymoquinone (TQ) has been reported as a potent inducer of apoptosis in cancer cells. However, the role of TQ as an apoptotic or antiapoptotic has not been established yet in other types of cell injuries. Our objective was to explore whether TQ exerts a hepatoprotective effect against hepatic ischemia reperfusion injury (I/R) and to identify its potential effect on apoptotic pathways. Rats were divided into eight groups: group I: sham-operated; group II: I/R (45 min ischemia-60 min reperfusion). The other six groups were given PO administration of TQ aqueous solution at 5, 20, and 50 mg/kg/day dose levels for 10 days. At the end of treatment three groups were not subjected to any intervention (groups III, IV, and V: TQ control groups) or subjected to 45 min ischemia followed by one-hour reperfusion as in group II (groups VI, VII, and VIII: TQ pretreated I/R groups). Serum levels of liver enzymes, tissue levels of malondialdehyde (MDA), reduced glutathione (GSH) and TNF-α were measured. Activities of caspases 8, 9, and 3 were determined. Cytochrome c in cytosol was determined by solid phase ELISA. Expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins as well as nuclear factor κB (NF-κB) were assessed using polymerase chain reaction. Apoptosis end point was detected using electrophoresis for analysis of DNA fragmentation. TQ administration before I/R resulted in a significant decrease in liver enzymes, MDA and TNF-α tissue levels with increased GSH content. It also inhibited cytochrome c release into the cytosol, down regulated the expression of NF-κB and Bax and up regulated the Bcl-2 proteins. Hepatic apoptosis was significantly attenuated as indicated by a significant decrease in all caspase activities and by DNA fragmentation. In conclusion, TQ exerts an antiapoptotic effect through attenuating oxidative stress and inhibiting TNF-α induced NF-κB activation. Furthermore, it regulates the Bcl-2/Bax ratio and inhibits downstream caspases in this I/R model.

Keywords: Thymoquinone (TQ), liver enzymes, malondialdehyde, reduced glutathione, caspases activities, cytochrome c, TNF-α, Bcl-2, Bax and NF-κB proteins expression, DNA fragmentation

1. Introduction

Recent evidence that apoptosis of hepatocytes is a feature in a wide variety of liver diseases including viral hepatitis, hepatocellular carcinoma and autoimmune diseases, has raised hopes that inhibition of apoptosis provides a new target for treatment of liver diseases (*1*).

Apoptosis or programmed cell death can be induced through the death receptor-dependent pathway or the mitochondrial-dependent pathway. The former pathway is initiated in the liver by death ligands like TNF and Fas ligands following binding to their relevant death receptors. This leads to caspase 8 activation with subsequent activation of down stream caspases such as caspase 3 (*2*). In contrast, the latter pathway is triggered by a variety of intracellular stressors such as DNA damage and hypoxic conditions. These stimuli cause the release of cytochrome c from mitochondria into the cytosol. Released cytochrome c, in the presence of dATP, forms an activation complex with

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apoptotic protein activating factor-1 and caspase 9 that activates down stream caspases to execute the final morphological and biochemical alterations. A group of antiapoptotic proteins tightly regulates the mitochondrial pathway, such as Bcl-2, and proapoptotic proteins such as Bax; further downstream regulation occurs, by various inhibitors of caspases (*3,4*).

Nigella sativa (NS) (black seed or black cumin) has been used traditionally to promote health and fight disease for centuries and most of the known biological activities of the seeds have been attributed to its active constituent thymoquinone (*5*). Recently conducted research has shown many therapeutic effects of TQ such as an immunomodulator, anti-inflammatory and antioxidant agent (*6*). Moreover, TQ is a potential chemotherapeutic compound owing to its antineoplastic activity against various tumor cells such as neoplastic keratinocytes, colorectal cancer cells and ovarian adenocarcinoma cells (*7-9*). The promising antineoplastic effect of TQ is attributed to its ability to induce apoptosis in cancer cells. Other studies demonstrated that TQ disrupts mitochondrial membrane potential and triggers the activation of caspases 8, 9, and 3 in myeloblastic leukemia HL-60 cells (*10*).

Although, all the mentioned studies support the antineoplastic role of TQ as a potent inducer of apoptosis in cancer cells, conflicting reports also showed evidence of delayed apoptosis upon treatment of Hep-2, laryngeal carcinoma cells, with the black seed (*11*). Other studies have shown lack of TQ toxicity to normal cells as well as its selective growth inhibitory and apopotic effect in cancer cells (*9,12*). As far as we know, the modulatory effect of TQ on apoptosis in normal cells and in other types of cell injuries has not yet been established. Here, we hypothesize a controversial effect of TQ that it could exert an antiapoptotic effect in another model of injury other than cancer. Therefore, we designed our study to compare the potential effect of TQ on apoptosis in normal rats and in a model of hepatic ischemia reperfusion injury. We studied the effect of TQ on various steps in the apoptotic pathway including death ligand, down stream caspase signaling of the apoptotic cascade and apoptotic regulators. New apoptosis-modulating compounds could be useful in the treatment of most liver disorders.

2. Materials and Methods

2.1. *Chemicals and reagents*

TQ (2-isopropyl-5-methyl-1,4-benzoquinone) was prepared as a 1 M stock solution in hot water (60~80°C) and the appropriate working solutions were prepared in a total volume of 1 mL. TQ, agarose, and DNA ladders were purchased from Sigma-Aldrich, Germany. EGTA (ethylene glycol tetraacetic acid) and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ellman's reagent, 5,5`-dithio bis-(2-nitrobenzoic acid, was from MP Biomedicals, Inc. (Washington, DC, USA). Liver enzyme kits were purchased from Quimica Clinica (Aplicada S.A., Spain). Cytochrome c release assay kit and TNF-α ELISA kit were products from Quantikine® (R&D Systems, Inc., USA). Caspases colorimetric assay kits were purchased from R&D Systems Inc., USA. Total protein quantification kit was made by Stanbio Lab (San Antonio, TX, USA). The RNA extraction kit was obtained from Promega, Madison, WI, USA. Reverse transcriptase (AMV), primers and Taq DNA polymerase, DNTPs were also from Promega. DNAeasy tissue kit was a product of Qiagen Inc., USA. All other reagents were of analytical grade.

2.2. *Animals*

Forty-eight Sprague Dawley male albino rats were purchased from the animal house of National Research Center, Cairo, Egypt. All animals were housed in plastic cages, kept in a conditioned atmosphere at 25°C and fed standard laboratory pellets with tap water *ad libitum*. All experimental procedures were carried out in accordance with the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication, 1985) (*13*).

2.3. *Experimental design*

Rats were randomly divided into eight groups; each consisted of 6 rats. Rats underwent either no intervention (group I: sham-operated group) or 45 min ischemia followed by one-hour reperfusion (group II: ischemia reperfusion injury group {I/R}). The other six groups were given PO administration of TQ aqueous solution at 5, 20, and 50 mg/kg/day dose levels for 10 days. At the end of treatment three groups were not subjected to any intervention (groups III, IV, and V: TQ control groups) or subjected to 45 min ischemia followed by one-hour reperfusion as in group II (group VI, VII, and VIII: TQ pretreated I/R groups).

2.4. *Surgical procedure*

Rats were anaesthetized using sodium phentobarbital at a dose of 30 mg/kg, 24 h after the last dose. A complete midline incision was made. The hepatoduodenal ligament was separated after entry into the belly (sham-operated group). The animal model of hepatic ischemia reperfusion injury was established according to Nauta *et al.* (*14*). To induce hepatic ischemia, the hepatic pedicle including hepatic artery and portal vein, which supplies the left and median liver lobes (70% of liver mass), was occluded with a microvascular clamp for 30 min. This method of partial hepatic ischemia allows for portal decompression through right and caudate lobes and so

prevents mesenteric venous congestion. Reperfusion was initiated by removal of the clamps. After one hour reperfusion, blood samples were collected from the retro-orbital plexus for biochemical analysis. Animals were scarified and livers were excised, cut into smaller pieces and stored at –80°C for analysis. The mitochondria were separated from the tissue soluble cytosolic fraction.

2.5. *Isolation of rat liver mitochondria and cytosol*

Rat liver mitochondria and cytosol were isolated by differential centrifugation as described by Johnson and Lardy (*15*). After the animals were killed, their livers were excised quickly and 0.5 g of each rat liver was placed in a medium containing 0.25 M sucrose, 10 mM Tris-HCl, and 1 mM of the chelator EGTA, pH 8 at 4°C. The tissue was scissor minced and homogenized on ice using a Teflon Potter homogenizer. Liver homogenate was centrifuged at 1,000 × g for 5 min to pellet cell fragments, and the supernatant was centrifuged at 9,500 × g for 10 min to pellet the nuclei. The supernatant was further centrifuged at 14,000 × g for 25 min to obtain the mitochondrial fraction and the resulting supernatant was used as the soluble cytosolic fraction.

2.6. *Determination of liver functions*

Determination of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to manufacturer's instructions using test reagent kits (Quimica Clinica Aplicada S.A., Spain). The analysis was performed on a Shimadzu UVPC 2401v 3.9 spectrophotometer (Shimadzu, Kyoto, Japan).

2.7. *Determination of reduced glutathione content (GSH)*

GSH content was measured in 10% liver homogenate according to the method of Ellman (*16*). Analysis was performed on a Shimadzu UVPC 2401v 3.9 spectrophotometer. GSH concentrations are expressed in mg/g tissue.

2.8. *Determination of lipid peroxides*

Lipid peroxidation was measured in 10% liver homogenate by the thiobarbituric acid (TBA) assay according to the method of Uchiyama and Mihara (*17*). Thiobarbituric acid reactive substances (TBARS) content was calculated according to the standard curve using 1,1,3,3-tetraethoxypropane as a standard and expressed in nmol/g wet tissue. The absorbance was measured at 535 nm using a Shimadzu UVPC 2401v 3.9 spectrophotometer.

2.9. *Determination of caspases activities*

Caspases (8, 9, and 3) activities were determined colorimetrically using assay kits (R&D Systems Inc, USA). Analysis was performed according to the manufacturer's instructions. 0.1 g liver tissue was added to 1 mL of the lysis buffer then homogenized on ice using the Teflon Potter homogenizer. After liver homogenate was centrifuged at 1,000 × g for 15 min the supernatant was suitable for the assay.

2.10. *Determination of cytochrome c in cytosol by solid phase ELISA*

Cytochrome c was measured in the soluble cytosolic fraction of liver homogenate after differential centrifugation as described above. Analysis was performed according to the manufacturer's instructions using Quantikine Rat/Mouse Immunoassay solid phase ELISA commercial kit. Cytosolic cytochrome c concentration was determined by optical density using the Vector multiple ELISA plate reader (Perkin-Elmer, USA) set to 450 nm. The cytochrome c values were expressed as ng/mg protein. Total protein analysis was performed using the total protein diagnostic kit.

2.11. *Determination of tumor necrosis factor α by solid phase ELISA*

Tumor necrosis factor α (TNFα) was determined by the Quantikine Rat/Mouse Immunoassay ELISA commercial kit according to the manufacturer's instructions. Zero point five gram liver tissue was added to 2 mL saline and the tissues were scissor minced and homogenized on ice using the Teflon Potter homogenizer. Liver homogenate was centrifuged at 1,000 × g for 15 min, and the supernatant was used for the assay. TNFα concentration was determined as optical density using the Vector multiple ELISA plate reader (Perkin-Elmer, USA) set to 450 nm. Values were calculated as pg/mL from the constructed standard curve, and then expressed as pg/mg tissue protein. Total protein analysis was performed using the total protein diagnostic kit.

2.12. *Detection of DNA fragmentation by gel electrophoresis*

Thirty mg liver tissue was homogenized using the Teflon Potter homogenizer. DNAeasy tissue kits were used for rapid tissue DNA extraction and purification following the manufacturer's instructions using the DNAeasy spin column. Five µL DNA samples were electrophoresed on 1.5% agarose, stained with ethidium bromide and visualized using a UV transilluminator (Uvitec, UK). DNA cleavage becomes evident in agarose gel electrophoresis and DNA cleavage results

in characteristic fragments of oligonucleosomal size (180-200 bp) (*18*).

2.13. *Determination of Bcl-2, Bax, and NF-κB genes expression using polymerase chain reaction (PCR)*

About 30 mg of liver tissues were homogenized in 175 µL lysis buffer containing guanidium thiocyanate and β-mercaptoethanol for RNA extraction.

2.13.1. *RNA extraction*

Total RNA was extracted after homogenization according to manufacturer's instructions. The concentration of extracted RNA was measured spectrophotometrically at 260 nm.

2.13.2. *Reverse transcription and polymerase chain reaction (RT-PCR)*

For amplification of the target genes, reverse transcription and PCR were run in two separate steps. Briefly, equal amounts of total RNA (10 µg) were heat denatured and reverse transcribed at 37°C for 60 min with 50 U/µL Moloney murine leukemia virus reverse transcriptase (MMLV-RT enzyme; Promega Co., Madison, WI, USA), 40 U/µL human placental ribonuclease inhibitor (HPRI) (Promega Co.), 10 mM deoxy-nucleoside 5'-triphosphate mixture (dNTPs), and 1 nM oligo-dT primer in a final volume of 30 µL of 1× MMLV-RT enzyme buffer. The reactions were terminated by heating at 95°C for 10 min and cooling on ice. The formed cDNA (5 µL) samples were amplified in 50 µL of 1× PCR buffer in the presence of 5 U/µL Taq DNA polymerase (Promega Co.), 1 µL 10 mM dNTPs, and 1 µL of the appropriate primer pairs (2 primers, 50 pmol each), and 37 µL DEPC water. The 3 sets of primers of Bax, Bcl-2, and NF-κB were designed from GenBank (accession No. G35510 and 691379, respectively); PCR consisted of a first denaturing cycle at 97°C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 3 min. A final extension cycle of 72°C for 10 min was included.

2.13.3. *Agarose gel electrophoresis*

All PCR products were electrophoresed on 2% agarose, stained with ethidium bromide and visualized by UV transilluminator.

2.13.4. *Semi-quantitative determination of PCR products*

β-Actin was used as an internal control and was also amplified using its specific primer. Semi-quantitation was performed using the gel documentation system (BioDO, Analyser) supplied by Biometra. According to the following amplification procedure, relative expression of each gene was calculated following the formula: R = densitometry units of each gene/ densitometry units of β-actin.

3. Results

3.1. *Effect of TQ on liver enzymes*

As it is shown in Table 1, I/R caused deterioration in liver functions as evidenced by a significant increase in AST and ALT activities as compared to sham-operated group ($p < 0.05$). Administration of TQ at doses of 5, 20, and 50 mg/kg for 10 days before I/R insult significantly reduced both AST and ALT activities as compared to the I/R group. The effect of 20 mg/kg TQ returned the AST activity to the sham-operated value ($p > 0.05$), while the 50 mg/kg TQ significantly decreased it below that of the sham-operated group ($p < 0.05$). The effect of TQ on liver enzyme activity in control rats was also studied (Table 2). We noticed that TQ significantly decreased the AST activity below that of the sham-operated group with no significant difference between groups III, IV, and V receiving different doses of TQ. For the ALT activity, TQ did not cause any significant change except for the dose of 20 mg/kg ($p < 0.05$ *vs*. sham group).

3.2. *Effect of TQ on lipid peroxidation*

As shown in Table 1, TQ markedly attenuated the increased MDA content, which is associated with hepatic I/R, in a dose dependent manner which reached

Table 1. Measurement of liver enzymes, MDA concentration, and GSH enzyme activities in TQ-IR pretreated groups

Groups	AST (U/L)	ALT (U/L)	GSH (mg/g)	MDA (nmol/g)
Control	87.90 ± 0.45	18.55 ± 0.54	2.47 ± 0.26	21.83 ± 0.80
I/R	141.96 ± 1.19[a]	65.90 ± 6.02[a]	0.58 ± 0.04[a]	63.4 ± 1.06[a]
VI	122.05 ± 1.05[a,b]	51.34 ± 3.73[a]	0.74 ± 0.02[a,b]	39.81 ± 1.88[a,b]
VII	90.08 ± 1.34[b]	29.83 ± 3.16[a,b]	0.99 ± 0.07[a,b]	30.56 ± 1.89[a,b]
VIII	75.16 ± 2.14[a,b]	22.76 ± 1.47[a,b]	1.49 ± 0.03[a,b]	11.03 ± 1.34[a,b]

Values are represented as mean ± SE, $n = 6$, [a] $p < 0.05$ *vs*. control, [b] $p < 0.05$ *vs*. I/R.

Table 2. Measurement of liver enzymes, MDA concentration and GSH enzyme activities in TQ-control groups

Groups	AST (U/L)	ALT (U/L)	GSH (mg/g)	MDA (nmol/g)
Control	87.90 ± 0.45	18.55 ± 0.54	2.47 ± 0.26	21.83 ± 0.80
III	64.53 ± 0.57[a]	17.85 ± 1.86	0.71 ± 0.06[a]	22.75 ± 1.18
IV	61.10 ± 0.68[a]	14.98 ± 0.68[a]	0.81 ± 0.05[a]	22.61 ± 1.54
V	62.46 ± 0.80[a]	16.67 ± 1.44	3.54 ± 0.03[a]	14.8 ± 0.76[a]

Values are represented as mean ± SE, $n = 6$, [a] $p < 0.05$ *vs*. control.

a significantly lower value than that of the sham group when the highest dose of 50 mg/kg ($p < 0.05$) was used. Results in Table 2 show that TQ at doses of 5 and 20 mg/kg, given to TQ control rats did not cause any significant reduction in MDA values as compared to the sham group ($p > 0.05$), while TQ at the 50 mg/kg dose significantly reduced the MDA content below that of the sham-operated group ($p < 0.05$).

3.3. *Effect of TQ on hepatic GSH content*

I/R caused a significant decrease in GSH ($p < 0.05$ *vs*. sham group) which was significantly elevated by TQ treatment in a dose dependent manner ($p < 0.05$ *vs*. I/R group). The increased level of GSH by TQ failed to return to sham-operated values for any of the doses used ($p < 0.05$). Interestingly, the antioxidant protective effect of TQ in the hepatic I/R model is reversed in the control groups of rats treated with TQ at doses 5 and 20 mg/kg. Results in Table 2 showed a significant decrease in the GSH level in groups III and IV as compared to the sham group ($p < 0.05$). On the other hand, the 50 mg/kg dose significantly increased the GSH level, which reached a higher level than the sham group ($p < 0.05$).

3.4. *Effect of TQ on caspases activities*

As shown in Figure 1, I/R induced apoptotic cell death was indicated by a significant increase in caspases 8, 9, and 3 activities. TQ administered at 5 mg/kg reduced apparently all caspases activities measured in the study but did not reach a significant level except for caspase 8. Meanwhile, TQ at doses of 20 and 50 mg/kg produced a dose-dependent decrease in caspase 3 (0.68 ± 0.05 and 0.50 ± 0.04, respectively; $p < 0.05$), caspase 8 (0.65 ± 0.04 and 0.40 ± 0.05, respectively; $p < 0.05$) and caspase 9 (0.63 ± 0.04 and 0.41 ± 0.05, respectively; $p < 0.05$) activities when compared to the I/R groups and values returned back to the corresponding sham operated situation with the 50 mg/kg dose of TQ. On the other hand, TQ did not cause any significant change in caspases 8, 9, and 3 activities at doses of 5 and 20 mg/kg in TQ control groups ($p > 0.05$ *vs*. sham). The 50 mg/kg TQ significantly reduced caspase 8 and 3 but had no effect on caspase 9 compared to the sham group (Figure 2).

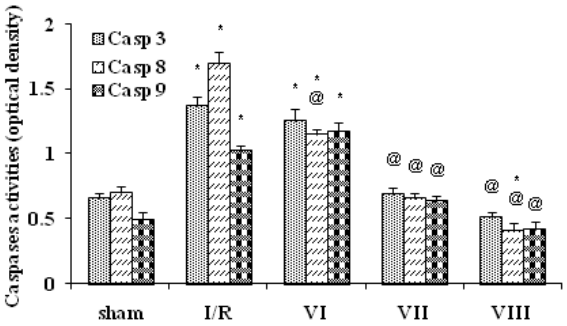

Figure 1. Effect of TQ on caspases activities in TQ-I/R groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control.

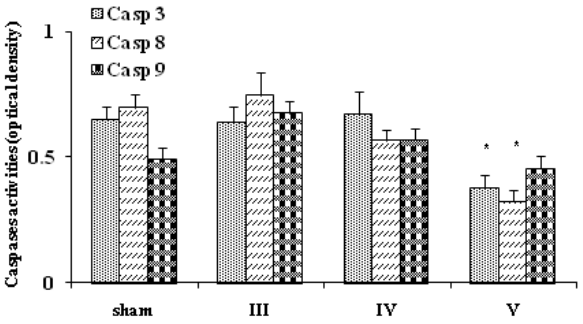

Figure 2. Effect of TQ on caspases activities in TQ groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control, @ $p < 0.05$ *vs*. I/R.

3.5. *Effect of TQ on cytochrome c*

As shown in Figure 3, I/R generated a significant increase in cytochrome c released into the cytosol as compared to the sham group ($p < 0.05$). TQ administration to TQ pretreated I/R groups VI, VII, and VIII resulted in a significant reduction in the released cytochrome c, in a dose dependent manner, as compared to the I/R group ($p < 0.05$). In TQ control groups III, IV, and V, no significant changes in the mean values of cytochrome c were detected as compared to the sham group ($p > 0.05$) (Figure 4).

3.6. *Effects of TQ on Bax and Bcl-2 protein levels*

As shown in Figures 5 and 11, I/R caused modulation of the apoptotic regulatory proteins towards apoptosis with a significant increase in the expression of proapoptotic

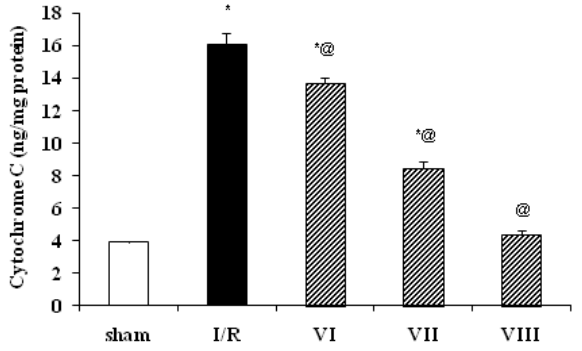

Figure 3. Effect of TQ on cytochrome C in TQ-I/R groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control, @ $p < 0.05$ *vs*. I/R.

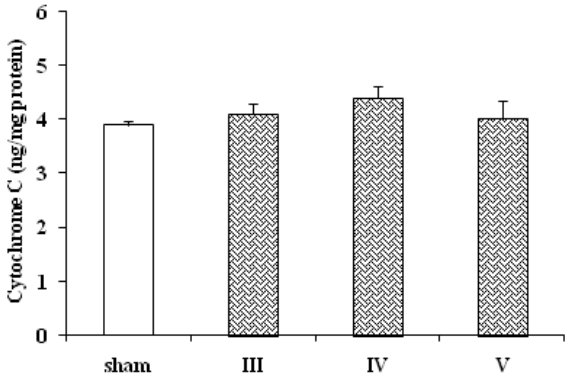

Figure 4. Effect of TQ on cytochrome C in TQ control groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control.

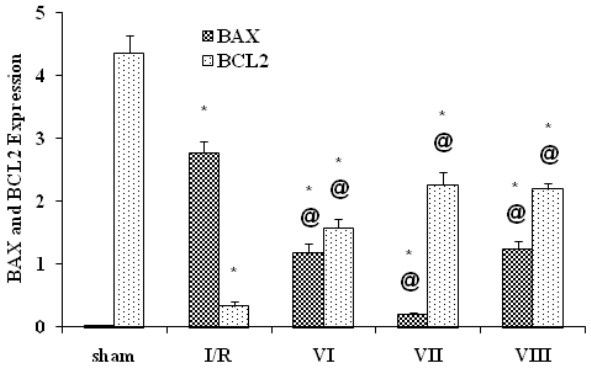

Figure 5. Effect of TQ on BAX and Bcl-2 proteins expression in TQ-I/R groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control, @ $p < 0.05$ *vs*. I/R.

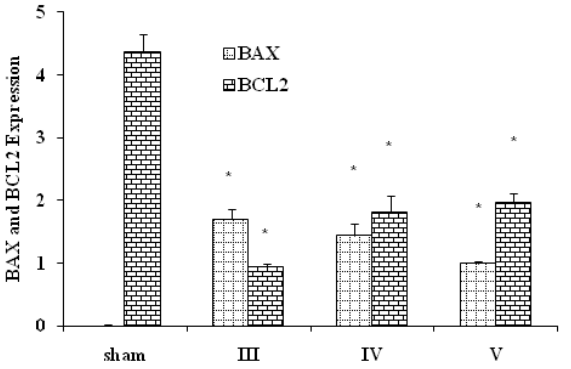

Figure 6. Effect of TQ on BAX and Bcl-2 proteins expression in TQ control groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control.

Bax protein and a significant decrease in the antiapoptotic Bcl-2 as compared to the sham group ($p < 0.05$). In groups VI, VII, and VIII receiving TQ at doses 5, 20, and 50 mg/kg, prior to I/R intervention, there was a significant decrease in Bax expression ($p < 0.05$) and a significant increase in Bcl-2 ($p < 0.05$). However, all doses of TQ used could not restore the Bax-to-Bcl-2 ratio to the control value ($p < 0.05$ *vs*. sham). On the other hand, administration of TQ to control rats in groups III, IV, and V resulted in a significant increase in the expression of Bax and a significant decrease in Bcl-2 as compared to the sham group ($p < 0.05$), raising the question whether TQ exerts an apoptotic effect on normal intact livers as shown in Figures 6 and 11.

3.7. *Effects of TQ on TNFα*

As shown in Figure 7, administration of TQ at doses of 20 and 50 mg/kg significantly reduced the TNFα levels compared to the I/R group. The hepatic content of TNFα was reduced from 0.79 ± 0.03 in the I/R group to 0.73 ± 0.04 and 0.58 ± 0.03 in groups VI and VII, respectively. The 50 mg/kg TQ given to group VIII returned the TNFα back to the control value in the sham-operated group ($p > 0.05$). Regarding the effect of TQ on TNFα in normal livers, TQ did not exert a significant effect in control rats at any of the doses used as illustrated in Figure 8 ($p > 0.05$ *vs*. sham group).

3.8. *Effects of TQ on NF-κB*

Figures 9 and 11 demonstrated the effect of TQ (5, 20, and 50 mg/kg) on NF-κB activation in the hepatic I/R model. TQ administered to rats at doses of 5 and 20 mg/kg prior to reperfusion injury significantly down regulated the increased expression of NF-κB by I/R ($p < 0.05$) but was still significantly higher than the control ($p < 0.05$). On the contrary, the 50 mg/kg dose did not cause any significant change in the NF-κB protein level as compared to the I/R group ($p > 0.05$). When control rats were treated with TQ, the expression of NF-κB was significantly increased at the 3 doses used in this study as compared to the sham group ($p < 0.05$), and opposite to the effect seen in the I/R model (Figures 10 and 11).

3.9. *DNA fragmentation*

Agarose gel analysis of fragmentation patterns of

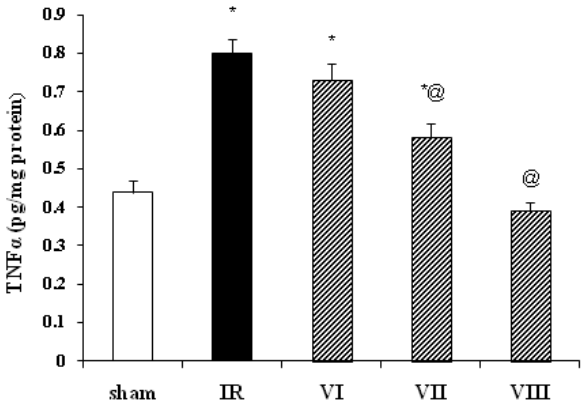

Figure 7. Effect of TQ on TNFα in TQ-I/R groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control, @ $p < 0.05$ *vs*. I/R.

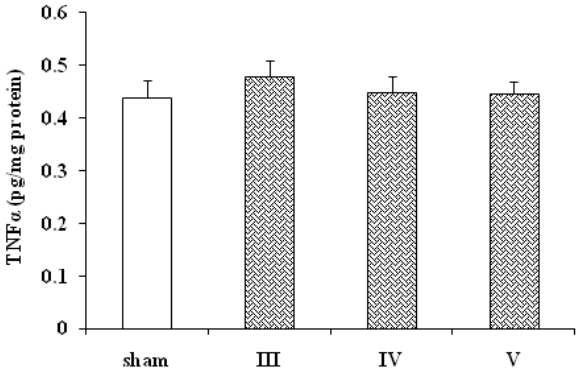

Figure 8. Effect of TQ on TNFα in TQ control groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control.

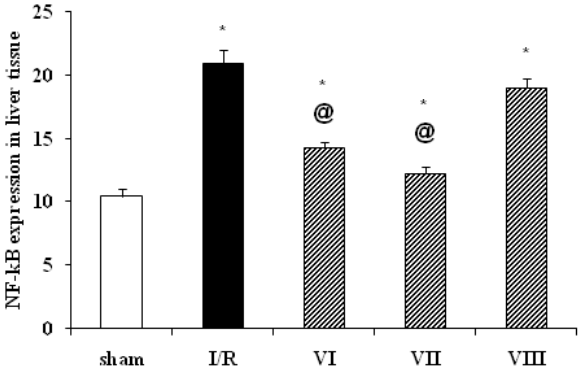

Figure 9. Effect of TQ on NF-κB in TQ-I/R groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control, @ $p < 0.05$ *vs*. I/R.

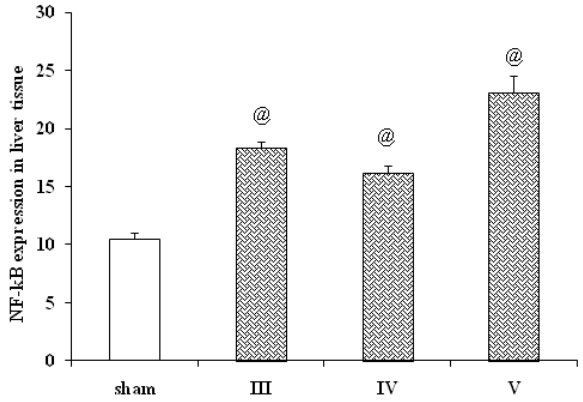

Figure 10. Effect of TQ on NF-κB in TQ control groups. Values are represented as mean ± SE, $n = 6$, * $p < 0.05$ *vs*. control.

cellular DNA isolated from I/R groups revealed the DNA ladder pattern of internucleosomal fragmentation. TQ pretreatment before the I/R insult decreased DNA fragmentation (Figure 12). Control groups treated with TQ were distinctly different from TQ pretreated I/R groups. There was a slight DNA fragmentation pattern only in TQ-control groups treated at 5 and 20 mg/kg doses while at the 50 mg/kg TQ dose, cellular DNA from rat group VIII showed no DNA ladder pattern when compared to the control group (Figure 13).

4. Discussion

Our results of TQ treatment on ALT and AST activities are in good agreement with a recent study by Yildiz *et al.* (*19*) who observed similar results with *Nigella* sativa (NS) extracts using the liver ischemic reperfusion model. In the present study, TQ treatment in groups VI, VII, and VIII significantly increased GSH levels in a dose dependent manner as compared to the I/R group. These results were consistent with the previous study on the effect of TQ on gastric mucosal I/R injury in rats. However, our

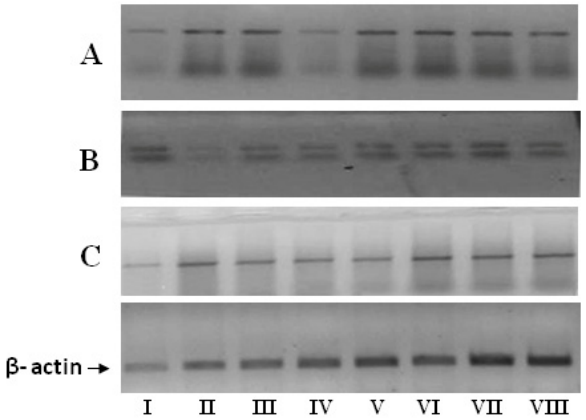

Figure 11. An agarose gel electrophoresis shows PCR products of BAX gene (A), BCL-2 gene (B), and NF-κB gene (C) in all animal groups (I-VIII).

results showed that GSH liver content in TQ control groups (III and IV) receiving 5 and 20 mg/kg of TQ were significantly decreased while the 50 mg/kg dose restored and significantly increased the GSH tissue content as compared to the sham-operated group ($p < 0.05$). This decrease in GSH content was reported in another *in vitro* study suggesting a TQ mediated-

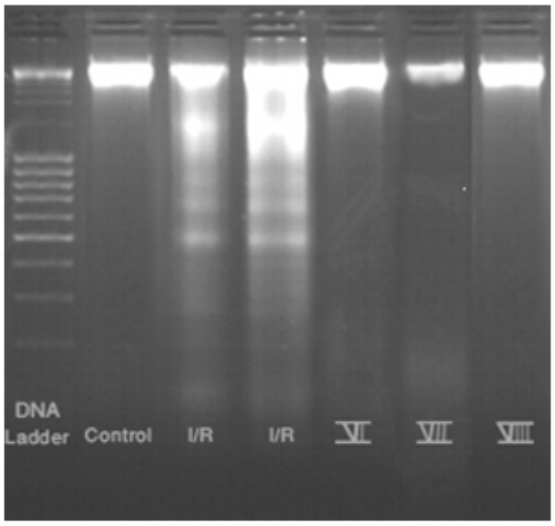

Figure 12. DNA fragmentation in TQ-I/R groups.

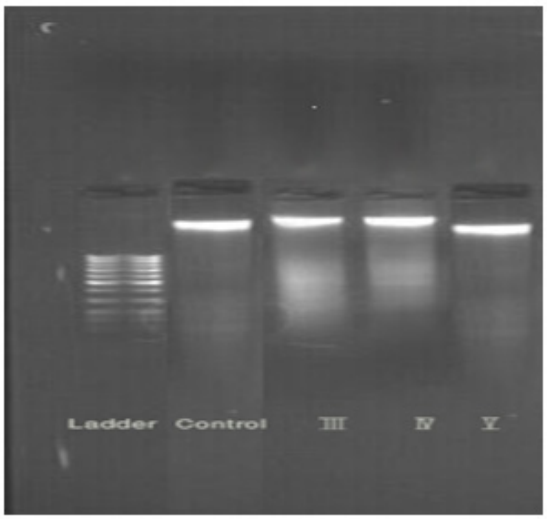

Figure 13. DNA fragmentation in TQ control groups.

GSH depletion effect in cancer cell lines (*20*). The effect of TQ on GSH content in different types of cell injuries is still questionable suggesting that the dose range used and the duration of treatment need further investigation.

The TQ effect on caspases cascade was first examined in the TQ control groups (Figure 2). Only the 50 mg/kg dose of TQ treatment caused a significant decrease in caspases 8 and 3 activities but had no effect on caspase 9 when compared to sham-operated group I. Therefore, the effect of TQ on caspases activities, in normal animal models, is only evident at the high dose and affected caspases 8 and 3. The TQ inhibitory effect on caspase 3 could be either through a direct effect or indirectly through deactivating up-stream caspase 8. The TQ negative effect on caspase 9 activities at all doses levels suggests that the TQ antiapoptotic effect in normal livers might be *via* targeting mainly inhibition of caspases 8 and 3 only. However, in TQ-I/R treated groups, we observed that the 20 mg/kg dose of TQ, resorted the caspases 8, 9, and 3 activities to normal values with complete protection against the I/R injury model especially with the 50 mg/kg dose. From these findings, we conclude that TQ inhibits downstream caspases and that caspase 8 is the most likely one targeted by TQ in the I/R model. This further supports our observations on the deactivation effect of TQ on caspase 8 in the normal animal groups.

TQ inhibited the release of mitochondrial cytochrome c into the cytosolic fraction in a dose dependent manner in all TQ-I/R groups compared to I/R groups but did not exert a significant change in the TQ control groups compared to the sham group. TQ deactivation of caspase 8 could be responsible for the inhibition of cytochrome c release from the mitochondria and thus the deactivation of caspase 3. The role of TQ and/or its metabolic reduced forms as free radical scavengers that result from I/R injury could lead to stabilization of the mitochondrial membrane permeability which regulates cytochrome c release and other caspase-induced pro-apopototic factors. This is further supported by our results in the present study where the gradual inhibition of lipid peroxidation measured as MDA hepatic tissue content are in agreement with the decrease of cytosolic cytochrome c levels in TQ pretreated groups VI, VII, and VIII. A conflicting *in vitro* study, conducted with isolated mitochondria from normal rat liver, showed the ability of TQ to induce mitochondrial O_2^- generation, suggesting that TQ enhances a mechanism of oxidative stress in mitochondria (*21*). However, in other studies, TQ has been reported to be a potent antioxidant that prevents oxidative injury in several tissues with the ability to inhibit lipid peroxidation and to preserve cell integrity (*22-24*).

This controversy could be explained by the ability of TQ to form a redox-couple consisting of the oxidized and semi-reduced forms of TQ similar to ubiquinone, a redox couple within the mitochondria, where the oxidized TQ becomes easily reduced and the semi-reduced form of TQ enhances O_2^- generation by accepting electrons from oxidizing species (*21*). In fact, these conflicting reports clarify the premise that the mechanism by which TQ induces changes in mitochondrial function *in vivo* might be different from that in *in vitro* studies.

Mitochondrial membrane permeability is tightly regulated by proteins from the Bcl-2 family which inhibit or promote mitochondrial membrane permeability depending on whether they belong to the pro- or anti- apoptotic branch of the family (*25-27*). Thus we determined changes in Bax and Bcl-2 protein expression levels upon TQ treatment. A significant increase in Bax protein with a similar decrease in Bcl-2 protein contents were found in all TQ-control

groups at all doses levels as compared to the sham-operated group. These data confirm previous results on the modulatory effect of TQ as proapoptotic in cancer cells. The reported mechanisms have been concluded in several studies to be p53-mediated where TQ induction of p53 was accompanied by down regulation of Bcl-2 (*8*). The Bax/Bcl-2 ratio changes, caused oligmerization of Bax on the outer mitochondrial membrane leading to permeabilization of the mitochondria with cytochrome c release (*28*). Interestingly our results showed that TQ causes up regulation of Bax and downregulation of Bcl-2 in normal animal groups, thus favoring the apoptotic pathway, while in the TQ-pretreated I/R groups, TQ caused a significant decrease in the Bax and concomitant increase in Bcl-2 levels as compared to the I/R group. However the values did not return to normal levels as in the sham-operated group. The mechanism is not fully understood and it needs further investigation. In a recent publication they reported upregulation of Bcl-2 using caspase 3 inhibitors to ameliorate ischemia reperfusion injury in a model of cardiac allograft in the rat. The inhibition of caspase 3 is concomitant with upregulation of Bcl-2 where caspase 3 inhibition mediates tryosine kinase activity allowing Bcl-2 overexpression (*29*). In the present study, results showed the inhibitory effect of TQ on caspase 3 activation at all doses levels in TQ pretreated I/R groups. Thus we might suggest that TQ deactivation of caspase 3 may allow for Bcl-2 overexpression that could provide partial protection against I/R injury-induced apoptosis. A distinct decrease in the DNA ladder pattern under TQ treatment supports our previous findings that TQ exerts an antiapoptotic effect against hepatic I/R injury.

Further findings on TQ's role in the death receptor pathway have to be highlighted (*30,31*). Ischemia reperfusion injury of the liver can lead to TNFα production, which can induce multiple mechanisms that initiate hepatocyte apoptosis (*32-34*). Bid, a proapoptotic protein, is activated by caspase 8 after TNFα binding to death receptor TNF-R1. The use of caspase 8 inhibitors to suppress Bid activation has been reported to inhibit cytochrome c release in TNFα-treated hepatocytes (*35*). This suggests the role of caspase 8 and the mitochondrial pathway in TNFα-induced apoptosis in hepatocytes. Further studies reported the role of TQ in attenuating pro-inflammatory responses *via* the inhibition of TNFα mRNA expression in a lipopolysaccaride-activated rat basophil cell line (*36*). In our present study TQ showed a significant dose dependent decrease in the TNFα content measured in liver homogenate of TQ pretreated I/R animal groups as compared to the I/R group. Thus we further suggest that the TQ anti-inflammatory effect could be through the deactivation of caspase 8, supporting the role of TQ administration in decreasing TNFα release in I/R injury. No distinct changes have been seen in all TQ-control groups as compared to the sham-operated group, indicating no effect of TQ on TNFα production under normal conditions.

On the other hand one of the multiple apoptotic pathways induced by TNFα has been linked to interference with the NF-κB signaling pathway. In our present study, we found that TQ at doses of 5 and 20 mg/kg induced a significant reduction in NF-κB expression in TQ pretreated I/R animal groups as compared to the I/R group, while the 50 mg/kg dose of TQ failed to cause any significant change in NF-κB expression.

The suppression in NF-κB values observed in groups administered TQ at doses of 5 and 20 mg/kg correlated with the TQ induced TNFα suppression in our hepatic I/R model. In agreement with our study, Sethi *et al*. investigated the effect of TQ on the NF-κB pathway. They found that TQ suppressed TNFα-induced NF-κB activation in a dose- and time-dependent manner and inhibited NF-κB activation induced by various carcinogens and inflammatory stimuli (*37*). Thus, we can conclude that the anti-apoptotic effect of TQ could be mediated in part through the suppression of TNFα and consequently suppression of the NF-κB activation pathway. On the other hand, the effect of TQ administration on the NF-κB expression protein levels in the normal animal model is opposite to its inhibitory effect in the I/R model. In TQ-control groups, TQ caused a significant NF-κB increase as compared to the sham-operated group. This increase is associated with a non detectable increase in TNFα content in TQ control groups, indicating that TQ activation of NF-κB is not only induced *via* the TNFα signaling pathway but also other pathways could be involved. Thus, it seems that in our study NF-κB activation observed in TQ-control groups induced an apoptotic effect especially because the Bax/Bcl-2 ratio in the TQ control groups is increased and the GSH level is depleted.

It can be concluded from this study that TQ exerts a potent antiapoptotic effect against hepatic I/R injury through attenuating oxidative stress and inhibiting TNFα-induced NF-κB activation. It inhibits caspases. It also inhibits cytochrome c release and targets the apoptotic regulators of the Bcl-2 family proteins. Concerning the effect of TQ on normal livers, TQ induced GSH depletion especially at the 5 and 20 mg/kg doses, increased the Bax to Bcl-2 ratio and activated transcription factor NF-κB, thereby promoting the apoptotic process. On the other hand, the 50 mg/kg dose of TQ decreased lipid peroxidation, increased the GSH hepatic content and inhibited caspase 8 and 3, favoring protection against apoptosis.

How TQ plays an anti-apopototic role in some conditions and a pro-apopototic in others requires further investigation.

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