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Original Article

Effect of Thymoquinone on Hepatic Fibrosis Induced in BALB/C Mice: Is It Always Beneficial?

Nancy S. Imam [a], Rehab Kamel [a,], Osama A. Badary [b] and Adel M. Mostafa [a, §]
[a] *Department of Pharmacology and Toxicology, Faculty of Pharmacy, Helwan University, Ein Helwan, Cairo, Egypt*
[b] *Department of Clinical Pharmacy, Faculty of Pharmacy, Ain Shams University, Alabbasseyah, Cairo, Egypt*
[§:] *Late Professor of Pharmacology and Toxicology, Faculty of Pharmacy, Helwan University*

A B S T R A C T

Thymoquinone (TQ) has a variety of beneficial properties including antioxidant and anti-inflammatory activities. In this study, the effect of TQ on biochemical changes and coagulopathy accompanying liver fibrosis in BALB/c mice was investigated. Fibrosis was induced by intraperitoneal injection of carbon tetrachloride (0.5 ml /kg) for 4 weeks. TQ was administrated at three dose levels (2, 4 and 8 mg/kg) in drinking water, starting 7 days before CCl_4 injection and continuing during the CCl_4 administration period (4 weeks).

CCl_4 raised serum alanine aminotransferase activity as well as hepatic malondialdehyde content and myeloperoxidase activity. This was accompanied by a significant decrease in superoxide dismutase, catalase activities and reduced glutathione level in liver. Treatment of mice with TQ ameliorated the hepatotoxicity induced by CCl_4, as evidenced by disappearance of fibrosis. Serum alanine aminotransferase activity, hepatic malondialdehyde content and myeloperoxidase activity decreased significantly while antioxidant enzymes activities rose. Prothrombin time and activated partial thromboplastin time were significantly elevated after injection of CCl_4 as compared to normal control group. TQ potentiated the effect of CCl_4 on the coagulation parameters.

Our results showed that TQ ameliorates hepatic fibrosis induced by CCl_4. However, it did not improve the coagulopathy induced in this model. Consequently, the use of TQ may be not always beneficial in different stages of chronic liver disease.

Key Words: Thymoquinone, Fibrosis, Ccl_4, Coagulopathy, Oxidative Stress.

Corresponding Author: Rehab Kamel **Email:** *kamelrehab@yahoo.com*

1. INTRODUCTION

Hepatic fibrosis results from a sustained wound healing in response to chronic liver injury **(Friedman, 2003).** It has multiple causes ranging from viral infections and alcohol abuse to autoimmune disease, non-alcoholic fatty liver disease and developmental anomalies **(Rebecca, 2006).** If liver is treated properly at stage of fibrosis, cirrhosis could be prevented **(Riley and Bhatti, 2001)**.

Decreased levels of most procoagulant factors and thrombocytopenia are the main haemostatic abnormalities associated with chronic liver disease. As a consequence, this condition was, until recently, considered as a prototype acquired coagulopathy. Little attention had been paid to the fact that, similar to procoagulant factors, their anticoagulant counterparts (namely protein C and antithrombin) are also reduced to the same extent. Therefore, the possibility that a rebalance of coagulation could take place in chronic liver disease has been ignored for many years (**Tripodi et al., 2011).**

Neubauer et al. have shown that fibrin accumulates in the liver during acute as well as chronic experimental liver injury **(Neubauer et al., 1995)**. Thrombin is responsible for the conversion of fibrinogen to fibrin. There are at least two mechanisms through which thrombin generation could be involved in fibrogenesis. Firstly, clot formation by itself leads to flow disturbance and local hypoxia which is a cofactor for fibrosis. Secondly, thrombin binds to several receptors that belong to the family of protease activated receptors (PAR). PAR-1 is upregulated in chronic injury leading to proliferation of hepatic

stellate cells and fibrous matrix deposition **(Duplantier et al., 2004; Marra et al., 1995).**

Carbon tetrachloride (CCl_4)-induced liver fibrosis model is similar to the human disease, from the standpoint of morphology and biochemical aspects of collagen metabolism **(Tamayo, 1983).** The hepatotoxic effect of CCl_4 is thought to result from its reductive dehalogenation by the CYP450 enzyme system to the highly reactive free radical, trichloromethyl radical ($CCl_3^{\cdot}$), leading to lipid peroxidation and activation of Kupffer cells, that is accompanied by the production of proinflammatory mediators **(McCay et al., 1984)**. Moreover, CCl_4 treatment results in nuclear factor kappa B (NF-κB) activation **(Liu et al., 1995)**. Activation of NF-κB has been shown to induce tissue factor (TF), a membrane-anchored protein acting as a cofactor for factor VIIa **(Bierhaus et al., 1995; Moll et al., 1995)** and involved in thrombin-mediated proliferation of cells **(Fischer et al., 1995).**

Previous studies demonstrated that Nigella sativa may be successful in the prevention of liver fibrosis in rabbits **(Turkdogan et al., 2001)**. Thymoquinone (TQ), the main constituent of the volatile oil obtained from Nigella sativa seeds, has been subjected to a range of pharmacological investigations in the past years. Its oral administration protected several organs against oxidative damage induced by a variety of free radical-generating agents. It is an efficient cytoprotective agent against hepatotoxicity by a combination of two mechanisms: decreasing hepatic lipid peroxidation **(Al-Gharably et al., 1997)** and preventing depletion of intracellular glutathione **(Daba and Abdel-Rahman, 1998).**

Using the CCl_4-induced liver fibrosis model, the aim of this study was to investigate the effect of TQ administration on fibrosis progression and coagulopathy accompanying it, and hence the merits or demerits of TQ use in chronic liver diseases.

2. MATERIALS AND METHODS

2.1. Drugs and chemicals

Thymoquinone and carbon tetrachloride were purchased from Sigma Aldrich Co (St. Louis, MO. USA). All the remaining chemicals were of the highest analytical grade.

2.2 Animals and experimental design

Adult BALB/c male mice, weighting 30-35 gm, were obtained from the breeding colony at the animal house of Theodor Bilharz Research Institute. Mice were housed in cages and provided free access to pelleted food and drinking water. The animal experiments described comply with the ethical principles and guidelines for the care and use of laboratory animals adopted by the National Egyptian Community.

BALB/c mice were divided into 8 groups (8 mice in each) as follows:

Group 1: mice received corn oil (5 µl/g i.p.) biweekly for 4 weeks.

Group 2: mice received CCl_4 (5 µl/g as a 1:9 mixture with corn oil) i.p. biweekly for 4 weeks.

Group 3: mice received TQ (2 mg/kg/day p.o) in drinking water for 5 weeks and were injected with corn oil (5 µl/g i.p.) biweekly starting from the second week and continuing during the TQ treatment period.

Group 4: mice received TQ (4 mg/kg/day p.o) in drinking water for 5 weeks and injected with corn oil (5 µl/g i.p.) biweekly starting from the second week and continuing during the TQ treatment period.

Group 5: mice received TQ (8 mg/kg/day p.o) in drinking water for 5 weeks and injected with corn oil (5 µl/g i.p.) biweekly starting from the second week and continuing during the TQ treatment period.

Group 6: mice received TQ (2 mg/kg/day p.o) in drinking water starting 1 week before injection of CCl_4 and during CCl_4 treatment period (4 weeks).

Group 7: mice received TQ (4 mg/kg/day p.o) daily in drinking water starting 1 week before injection of CCl_4 and during CCl_4 treatment period (4 weeks).

Group 8: mice received TQ (8 mg/kg/day p.o) daily in drinking water starting 1 week before injection of CCl_4 and during CCl_4 treatment period (4 weeks).

Twenty four hours after the last corn oil or CCl_4 injection, blood samples were taken from mice by retro-orbital puncture. A part was collected on 3.8% trihydrated trisodium citrate solution and centrifuged at 3000 rpm for 5 min. The obtained clear non-haemolysed plasma was used for estimation of prothrombin time (PT) and activated partial thromboplastin time (aPTT).

The other part of blood sample was left to clot at room temperature and centrifuged at 3000 rpm for 5 min in order to obtain a clear non- haemolysed serum. The serum was used for estimation of liver function parameters.

After the collection of the blood samples all mice were sacrificed by cervical dislocation. The liver was rapidly removed, blotted dry and weighed. Samples of the liver were fixed with 10% formal saline for histopathological study. Liver sample from each mouse was homogenized in ice cold normal saline to obtain a 10% (w/v) homogenate. The

homogenates were centrifuged at 10000 rpm for 30 min at 4°C and supernatants were stored at −70°C until biochemical assays could be performed.

2.3. Determination of serum alanine aminotransferase (ALT) activity

The serum ALT activity was determined by standard spectrophotometric procedure as described by **Reitman and Frankel (1957)** using commercially available diagnostic kit supplied by Quimica Clinica Aplicada (S.A., Spain).

2.4. Determination of lipid peroxide level

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) level in the liver homogenate supernatants according to **Mihara and Uchiyama (1978)**.

2.5. Determination of reduced glutathione (GSH) content

Liver homogenate content of GSH was measured according to method of **Ellman (1957)**.

2.6. Determination of antioxidant enzymes activities

2.6.1. Determination of Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined by calculating the difference between autooxidation of pyrogallol alone and in the presence of homogenate that contained SOD according to the method described by **Marklund (1985)**.

2.6.2. Determination of Catalase Activity

Catalase (CAT) activity was determined based on the decrease in light absorption at 240 nm because of the decomposition of hydrogen peroxide by catalase **(Claribone, 1985)**.

2.7. Determination of myeloperoxidase (MPO) activity

MPO activity was measured in liver homogenate according to method described by **Pinegin et al. (1995)**.

2.8. Determination of total protein

Total protein of liver homogenate supernatants was determined according to **Lowry et al. (1951)**.

2.9. Determination of coagulation parameters

Determination of PT and aPTT was done using test reagent kits (DiaMed AG, Cressier sur Morat, Switzerland) according to the methods described by **Quick et al. (1935)** and **Bell and Alton (1954)**, respectively.

2.10. Histopathological examination of liver sections

Autopsy samples were taken from the liver of rats in different groups then fixed in 10% formal saline. Serial dilutions of alcohol (methyl, ethyl, and absolute ethyl) were used. Specimens were cleared in xylene embedded in paraffin at 56◦C in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 μm thickness by a sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin and eosin stain for histopathological examination through the light microscope.

2.11. Statistical analysis

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

3. RESULTS

3.1. Serum ALT activity, hepatic MDA and GSH content

We examined the hepatoprotective effect of TQ on CCl_4-induced fibrosis in mice. CCl_4 injection increased the serum alanine aminotransferase (ALT) activity by 22-folds, compared with control group. Administration of TQ at doses of 2, 4 and 8 mg/kg to mice treated with CCl_4 significantly reduced serum ALT activity by approximately 19%, 46% and 42%, respectively, as compared to the CCl_4 treated group.

Hepatic malondialdehyde (MDA) level increased significantly in the CCl_4- injected mice by 150% as compared to the control group. Administration of 4 and 8 mg/kg of TQ to CCl_4 treated group reduced significantly hepatic MDA content by approximately 32% and 29%, respectively, as compared to the CCl_4 treated group.

On the other hand, hepatic glutathione content in the CCl_4-injected mice decreased by almost 42.51% compared to mice treated with corn oil. Administration of TQ at a dose of 2 mg/kg to mice treated with CCl_4 significantly increased hepatic GSH content by approximately 55%, while 4 and 8 mg/kg of TQ significantly increased hepatic GSH by approximately 2 folds as compared to the CCl_4 treated group (Table 1).

3.2. Antioxidant enzymes and myeloperoxidase activities

As shown in Table 2, SOD and CAT activities were significantly decreased by 46.52% and 45.18%, respectively, after CCl_4 injection as compared to the corn oil control group. TQ (2 mg/kg) did not

significantly change SOD and CAT activities. The other doses (4 and 8 mg/kg) significantly increased SOD activity by 45.9% and 42.8%, respectively, while CAT activity was increased by 31.8% and 29.55%, respectively, as compared to the CCl_4 treated group. Concerning hepatic MPO activity, CCl_4 augmented it by approximately 70.52% as compared to the corn oil group. Administration of TQ at doses of 2, 4 and 8 mg/kg to mice treated with CCl_4 significantly decreased hepatic MPO activity by approximately 26.5%, 37% and 34%, respectively, as compared to the CCl_4 treated group.

Administration of different doses of TQ to corn oil treated group did not change the previous parameters measured in serum or liver homogenate compared to the control group (results not shown).

3.3. Coagulation parameters

Administration of TQ at doses of 2, 4 and 8 mg/kg to BALB/c mice significantly increased PT value by approximately 26.5%, 30.17% and 29.39% respectively as compared to the control group. Similarly, aPTT value increased by 24.13%, 31.55% and 29.07% respectively as compared to the control group (Figure 1).

Intraperitoneal injection of CCl_4 significantly increased PT and aPTT values by approximately 41.11% and 46.03%, respectively, as compared to the control group.

Administration of TQ at a dose of 2 mg/kg to mice treated with CCl_4 did not significantly change PT value while it increased aPTT value by 9% as compared to the CCl_4 treated group. TQ at doses of 4 and 8 mg/kg increased PT value by approximately 17%, while they increased aPTT value by approximately 21% and 18%, respectively, as compared to the CCl_4 treated group (Figure 2).

3.4. Histopathological examination of liver sections

Liver section from corn oil control mice showed normal morphological and histological features without inflammation or necrosis (Figure 3 A). In contrast, the liver injured by chronic CCl_4 administration displayed an obvious infiltration of inflammatory cells (M) along with necrotic damages (N) and appearance of focal area of fibrosis (F) (Figure 3 B)**.** Treatment with TQ (2 mg/kg) showed karyomegalocytes of the hepatocytes (k), degenerated hepatocytes (d), and few inflammatory cells infiltration in between the hepatocytes (Figure 3 C)**.** Administration of TQ (4 mg/kg) to the CCl_4 treated mice showed a liver with vacuolar degeneration in the cytoplasm of the hepatoytes (Figure 3 D), while TQ (8 mg/ kg) showed a liver with Kupffer cells proliferation (arrow) in between the degenerated cytomegalic hepatocytes (arrow) (Fgure 3 E). We did not notice any fibrous accumulation in the groups treated with different doses of TQ.

Table (1): Effect of thymoquinone (TQ) (2, 4 and 8 mg/kg) treatment on serum alanine aminotransferase (ALT) activity, hepatic malondialdehyde (MDA) and reduced glutathione (GSH) levels in CCl_4 induced liver fibrosis in BALB/c mice.

Groups	ALT (U/L)	MDA (nmol/g tissue)	GSH (µmol/g tissue)
Corn oil (Control)	36.96 ± 0.05	285.51 ± 23.26	4.41 ± 0.20
CCl_4	808 ± 4.11 [a]	713.04 ± 31.68 [a]	2.54 ± 0.14 [a]
TQ2+CCl_4	652.5 ± 3.22 [a,b]	687.74 ± 29.60 [a]	3.94 ± 0.186 [b]
TQ4+CCl_4	436.25 ± 6.88 [a,b,c]	483.72 ± 18.8 [a,b,c]	5.66 ± 0.25 [a,b,c]
TQ8+CCl_4	470 ± 10.20 [a,b,c,d]	505.71 ± 26.53 [a,b,c]	6.06 ± 0.33 [a,b,c]

Results are expressed as mean ± SE, n= 8 mice/group.
Different groups were compared using one way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.
a: significantly different from control group at $p < 0.05$.
b: significantly different from CCl_4 group at $p < 0.05$.
c: significantly different from TQ2+CCl_4 group at $p<0.05$
d: significantly different from TQ4+CCl_4 group at $p<0.05$

Table (2): Effect of thymoquinone (TQ) (2, 4 and 8 mg/kg) treatment on hepatic superoxide dismutase (SOD), catalase (CAT) and myeloperoxidase (MPO) activities in CCl_4 induced liver fibrosis in BALB/c mice.

Groups	SOD (U/mg protein)	CAT (U/mg protein)	MPO (U/mg protein)
Corn oil (Control)	24.4 ± 2.93	17.71 ± 0.36	13.3 ± 0.29
CCl_4	13.05 ± 0.72 [a]	9.71 ± 0.39 [a]	22.68 ± 0.89 [a]
TQ2+CCl_4	16.05 ± 0.78 [a]	10.42 ± 0.19 [a]	16.66 ± 1.17 [b]
TQ4+CCl_4	19.04 ± 0.60 [a,b]	12.80 ± 0.36 [a,b,c]	14.3 ± 0.26 [b]
TQ8+CCl_4	18.64 ± 0.54 [a,b]	12.58 ± 0.20 [a,b]	15 ± 0.31 [b]

Results are expressed as mean ± SE, n= 8 mice/group.
Different groups were compared using one way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.
a: significantly different from control group at $p < 0.05$.
b: significantly different from CCl_4 group at $p < 0.05$.
c: significantly different from TQ2+CCl_4 group at $p<0.05$

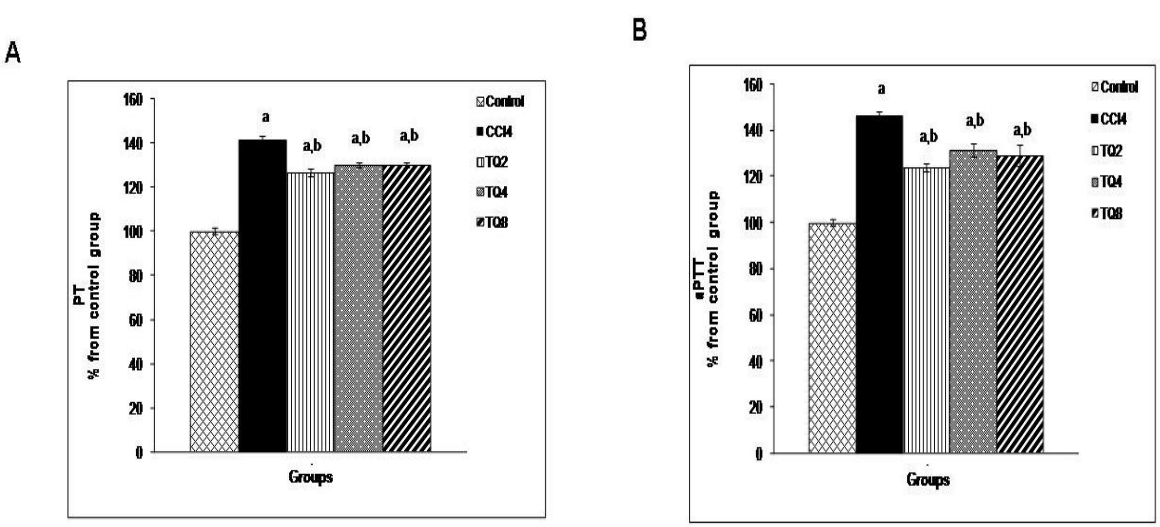

Figure 1. **Effect of thymoquinone (TQ) treatment on prothrombin time (PT) and activated partial thromboplastin time (aPTT) values in mice injected with corn oil.** Results are expressed as percentage of control group. n= 8 mice/group.
Different groups were compared using one way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.
a: significantly different from control group at $p < 0.05$.
b: significantly different from CCl_4 group at $p < 0.05$.

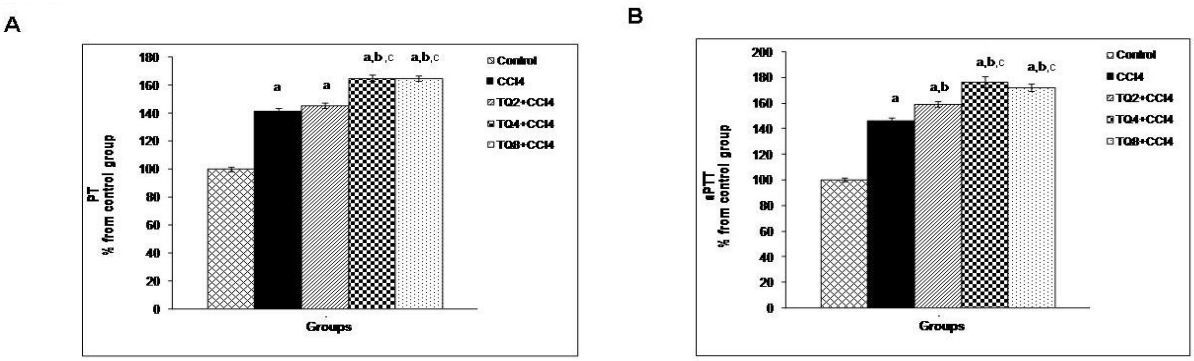

Figure 2. **Effect of thymoquinone (TQ) treatment on prothrombin time (PT) and activated partial thromboplastin time (aPTT) in mice with CCl_4-induced liver fibrosis.** Results are expressed as percentage of control group. n= 8 mice/group.

Different groups were compared using one way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

a: significantly different from control group at $p < 0.05$.

b: significantly different from CCl_4 group at $p < 0.05$.

c: significantly different from TQ2+CCl_4 group at $p<0.05$

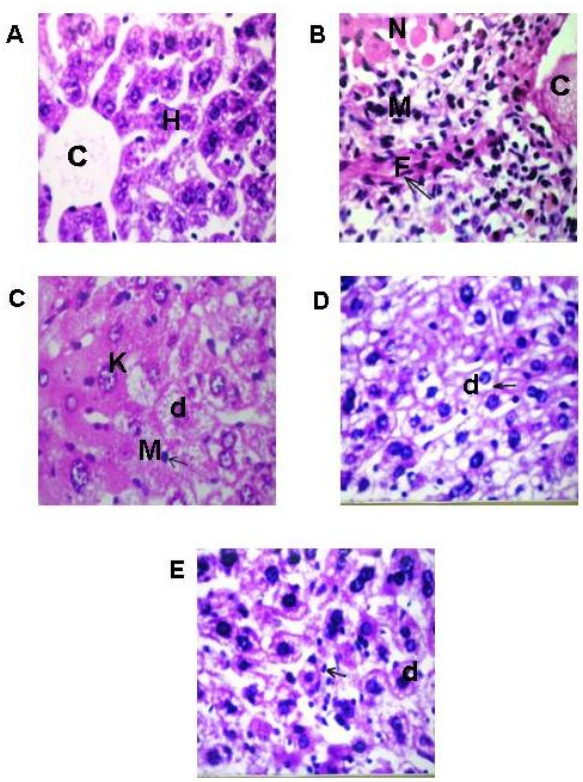

Figure 3. Histopathological examination of liver sections

Original magnification X 160.

(A) Corn oil treated mice. **(B)** CCl_4 treated group showing fibrosis (F) with inflammatory cells infiltration (M) replacing the necrosed tissue. **(C)** TQ2 (2 mg/kg) treated group showing karyomegalocytes of the hepatocytes (k), degenerated hepatocytes (d), inflammatory cells infiltration in between the hepatocytes (arrow) and leukocytes impacted the central vein (c)**. (D)** TQ4 (4 mg/kg) treated group showing vascuolar degeneration in the cytoplasm of the hepatocytes**. (E)** TQ8 (8 mg/kg) treated group showing Kupffer cells proliferation (arrow) in between the degenerated cytomegalic hepatocytes (arrow)**.**

4. DISCUSSION

The current study aimed to investigate the effect of TQ on biochemical changes and coagulopathy accompanying fibrosis induced by CCl_4 injection in BALB/c mice.

Treatment with CCl_4 resulted in a significant increase in serum ALT activity. Lipid peroxidation measured as MDA content in liver tissue was increased. CCl_4-induced lipid peroxidation may account for the release of intracellular enzymes such as amino transferases into the extracellular compartments, including blood serum **(Zimmerman, 1978).**

We found also that hepatocellular injury provoked by administration of CCl_4 caused a decrease of hepatic GSH. This depletion was explained on the basis that CCl_4, is hydrolyzed by CYP450 and activated to the trichlorometlyl radical ($CCl_3^{\cdot}$) which is conjugated by GSH that detoxifies this toxic metabolite and facilitates its excretion. Accordingly, exhaustion of intracellular substances such as glutathione, which is capable of preferentially conjugating with the toxic metabolites, is one of the sequences of CCl_4-induced injury **(Burk et al., 1983; Szymonik-Lesiuk et al., 2003).**

Similarly, SOD and CAT activities were significantly decreased by injection of CCl_4. This finding is in accordance with **Ko and Lim (2006)** and can be explained on the basis that SOD and CAT are part of the enzymatic antioxidant defense systems that have been developed by cells to cope up with the ROS and other free radicals. When a condition of oxidative stress establishes, the defense capacities against ROS becomes insufficient **(Wei, 1998).**

Free radicals generated by CCl_4 metabolism activate Kupffer cells. Neutrophils are attracted and activated by the cytokines secreted by the activated Kupffer cells. Infiltration of neutrophils into tissues is commonly assessed by changes in activity of myeloperoxidase (MPO), an enzyme found primarily in neutrophils that produce hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) and chloride anion (Cl^-). In this study, we observed an increase of the MPO activity in the liver tissues of mice treated with CCl_4 which may indicate that neutrophil accumulation contributes to CCl_4-induced liver injury. This finding was confirmed by the histopathological study showing increased number of inflammatory cells.

In the present work, administration of TQ one week before and during CCl_4 injection period elicited hepatoprotective effect. This was demonstrated by the significant decrease in ALT activity at different doses (2, 4 and 8 mg/kg). These doses prevented depletion of intracellular GSH. The elevation of hepatic GSH level observed in CCl_4 groups treated with TQ may be explained by the ability of TQ to induce antioxidant enzymes like quinone reductase in mice liver **(Nagi and Almakki, 2009; Prestera et al., 1993)**. Prevention of GSH depletion by TQ leading to maintenance of cell membrane integrity may explain the reduction of serum ALT leakage **(Daba and Abdel-Rahman, 1998)**.

Concerning the effect of TQ on hepatic antioxidant enzymes, we found that treatment of CCl_4-injected mice with TQ (2 mg/kg) did not significantly change SOD and CAT activities, while the other doses (4 and 8 mg/kg) significantly increased enzymes activities. This increase could presumably be explained as a consequence of the decrease in these enzymes substrates (superoxide radical or free radicals) due to the reported superoxide radical scavenging effect of TQ **(Badary et al., 2003)**. Another study demonstrated that TQ supplementation increases the expression of antioxidant genes like superoxide dismutase, catalase and glutathione peroxidase in rat liver **(Ismail et al., 2009).** Thus, TQ may reduce oxidative stress through free radical scavenging effect as well as induction of endogenous antioxidant enzymes expression leading to the prevention of CCl_4-induced hepatotoxicity. This is further supported by the reduction of lipid peroxidation, measured as MDA hepatic content, by 4 and 8 mg/kg of TQ.

In addition to its antioxidant effect, we found that different doses of TQ used in this study reduced inflammation induced by CCl_4 administration. This was reflected by the decrease in MPO activity. Our finding is in accordance with previous studies proving the anti-inflammatory effect of TQ **(Tekeoglu et al., 2007**; **Sethi et al., 2008; Lei et al., 2012).**

Administration of TQ (2, 4 and 8 mg/kg) prolonged PT and aPTT compared to the oil control group. This can be explained on the basis of structural similarity of TQ to benzoquinones which act as tissue factor/VIIa (TF/VIIa) complex inhibitors **(Abdel-Fattah et al., 2000; Parlow et al., 2003).** TF/VIIa complex activates factors IX (one of the factors involved in the intrinsic pathway) and X (one of the factors involved in the common pathway) to IXa and Xa respectively. Factor Xa participates in conversion of prothrombin to thrombin, leading to fibrin formation, deposition and subsequent thrombus formation **(Houston, 2002).** Inhibition of TF/VIIa complex may result in the decrease of thrombin, which has a prominent role in the coagulation cascade and in activating platelet aggregation. In addition, it is also recognized to act as an activator of hepatic

stellate cells, the mediators of hepatic fibrogenesis, via PAR1 receptors. The possible decrease in thrombin formation may explain the disappearance of fibrosis in the liver sections of CCl_4-injected groups treated with TQ (2, 4 and 8 mg/kg). Administration of CCl_4 was found also to prolong PT and aPTT values significantly when compared to the oil control group. This may be explained on the basis of deficiency of blood coagulation factors due to decrease in the synthesizing activity of the parenchymal cells and cells necrosis caused by administration of CCl_4. These results are in accordance with **Okazaki et al. (1986)**, **Fujiwara et al. (1988) and Hirata et al. (1989).** Therefore, CCl_4 seemed to induce extrinsic and intrinsic coagulation factors deficiency. Administration of TQ to CCl_4- injected groups significantly increased PT and aPTT as compared to the CCl_4 treated group. So TQ potentiated the coagulopathy produced by CCl_4 administration. This effect may cause a hindrance to the use of TQ in case that procoagulant factors are diminished due to chronic liver diseases.

In conclusion, our work showed the ability of TQ to protect liver from chemically-induced fibrosis due to its antioxidant and anti-inflammatory effects. However, it may worsen the coagulopathy associated with fibrosis. This finding pointed to that TQ may not be always beneficial in all cases of chronic liver diseases. Further studies will be needed to investigate the mechanistic effect of TQ on blood coagulation.

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