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Thymoquinone is protective against 2,3,7,8-tetrachlorodibenzo-p-dioxin induced hepatotoxicity

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Abstract

We investigated changes in rat liver tissues following administration of thymoquinone (TQ) against 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced hepatotoxicity. Fifty rats were assigned randomly to five groups of 10 as follows: control, corn oil, TCDD, TQ and TCDD + TQ. Biochemical and histopathological analyses were conducted on liver tissue. We found that 30 day TCDD administration caused histopathological changes in liver including thickening of Glisson's capsule, intracytoplasmic vacuolization in hepatocytes, sinusoidal dilation, vascular and sinusoidal congestion and inflammatory cell infiltration. TCDD administration increased malondialdehyde (MDA), total oxidant status (TOS), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels in rat liver tissue and reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and total antioxidant status (TAS) levels compared to all other groups. In the TQ treated group, GSH, SOD, CAT and TAS levels increased compared to all other groups. MDA, TOS, ALT, AST, ALP levels decreased compared to all other groups. Our histological findings were consistent with the biochemical findings. The oxidative and histologic effects of TCDD were eliminated by TQ treatment. TCDD administration caused oxidative stress in rat liver and TQ administered with TCDD prevented TCDD induced hepatotoxicity. TQ could be considered an alternative anti-TCDD toxicity agent.

Key words: hepatotoxicity, liver, oxidative stress, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, thymoquinone

Dioxin is the general name for a group of compounds that contain two chlorinated benzene rings linked by two oxygen bridges. They are produced primarily by chlorophenols that are used as fungicides, insecticides and bactericides; in paper manufacturing and pharmaceutical preparations; and for preservation of raw materials for medical, dental and cosmetic products. Owing to their resistance to environmental conditions and their high oil/water distribution coefficient, dioxins can accumulate in high concentrations in organisms that are exposed to them. Because they are fat soluble, they enter the food chain and accumulate especially in predators.

The most toxic dioxin is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Ministry of Health and Welfare (Japan) 1996, Pohjanvirta and Tuomisto 1994, Shen et al. 2016, Zhang et al. 2013). In general, the toxic effects of TCDD include developmental disorders, thymic atrophy, epithelial

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disorders, liver damage, immune deficiency, cancer, alteration of steroidogenesis (Jonosek et al. 2005) and decreased expression of sex hormones and LH receptors (Riddick et al. 2004) due to increased expression of CYP1A1, CYP1A2, CYP1B1 and other CYP genes in the liver. The damaging effects of dioxins also include lipid oxidation (Miranda et al. 2006). Owing to the increased synthesis of stoichiometric P450 enzymes by dioxin compounds, molecular oxygen transport increases, which causes formation of reactive oxygen species and lipid oxidation (Parzefall 2002, Bentli et al. 2016). Endogenous antioxidants are either enzymatic or non-enzymatic. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione-S transferase (GST). Non-enzymatic antioxidants include non-protein sulfhydryls including reduced GSH; N-acetylcysteine; thiols and carotenoids; vitamins A, C, E; taurine; melatonin and uric acid among others (Aydın et al. 2001, Mochida et al. 2007). Oxidative stress reflects an imbalance between free radical formation and the antioxidant defense mechanism, which results in tissue damage (Serafini and Del Rio 2004).

Herbal medicines are used increasingly for treatment of various diseases. *Nigella sativa* has been used for hundreds of years as a health supplement and for treating illness (Salem 2005). The biological activities and chemical components of *N. sativa* have been studied extensively. Thymoquinone (TQ), the active ingredient in *N. sativa*, is found in the essential oil of the seed. *Nigella* seeds and components have been reported to possess anti-carcinogen, antitumor (Kaseb et al. 2007, El Khoely et al. 2015), antibacterial (Jaswal et al. 2013), anti-inflammatory, antioxidant and immune system enhancing effects (Galaly et al. 2014, Mabrouk 2017). We examined the effects of TQ on liver tissue of rats exposed to TCDD toxicity.

Material and methods

Experimental animals

We used 50 250 ± 20 g 5-month-old male Wistar albino rats that were procured from Inonu University Faculty of Medicine, Experimental Animal Production and Research Center (INUTF-DEHUM). The study was approved by the experimental animals ethics committee (2017/A-49). Fresh drinking water was provided for the animals and they were fed *ad libitum* with standard pellet feed. The cages were cleaned daily during the experiment. The rats were housed in rooms at 21° C, 55 – 60% ambient humidity and a 12 h light (08:00 to 20:00 h):12 h dark cycle.

Experimental design

The 50 Wistar rats were assigned randomly to five groups of 10. Group 1, control (C), was given physiological saline. Group 2, corn oil group (CO), was given corn oil. Group 3, (TCDD), was given 1 µg/kg TCDD (0001746016; Sigma Aldrich, St. Louis, MO) (Boverhof et al. 2005). Group 4, (TQ), was given 50 mg/kg TQ (0000490915; Sigma Aldrich) (Kong et al. 2015). Group 5, (TCDD + TQ), were given 1 µg/kg TCDD and 50 mg/kg TQ.

TCDD was dissolved in corn oil and TQ was dissolved in saline; 1 ml doses were administered to each rat by gavage. The solutions were administered for 30 days at the same time every day.

Samples

At the end of the experiment, rats were decapitated under xylazine-ketamine anesthesia and liver tissue samples approximately 1 × 0.5 x 0.3 cm were obtained. The samples were cleaned with saline. Blocks of tissues were placed in 10% formaldehyde for fixation for histopathological examination; the remaining portion was stored at −80° C for biochemical analyses.

Preparation of tissues for biochemical analysis and measurement

Frozen liver tissues were thawed and weighed on the day of the experiment. Phosphate buffer was added to form a 10% homogenate and the product was homogenized at 12,000 rpm for 1 – 2 min in ice (IKA, Staufen, Germany). Tissue homogenates were centrifuged at 600 x g at 4° C for 30 min to obtain the supernatant.

Malondialdehyde (MDA) analysis was performed using the method reported by Uchiyama and Mihara (1978). MDA concentration was determined by measuring the absorbance at 535 and 520 nm at 95° C of the supernatant that was extracted from the n-butanol phase of the pink colored product of the reaction between the MDA and thiobarbituric acid.

GSH analysis was conducted using the method described by Ellman (1979). GSH concentration

was determined by measuring the absorbance of the yellow-green color produced by the reaction between GSH and 5,5′-dithiobis 2-nitrobenzoic acid at 410 nm in the spectrophotometer.

Total reduction of nitro blue tetrazolium by the superoxide anion, a product of xanthine and xanthine oxidase, reflects SOD activity (Jolitha et al. 2006). The quantity of protein that inhibited the NBT reduction rate by 50% was defined as the SOD activity unit and the results were reported in U/mg protein.

Total protein content in liver supernatant was determined using the method of Lowry et al. (1951). Concentrated Coomassie blue (Bio-Rad, Hercules, CA) was diluted 1:5 (v/v) with distilled water, then 2.5 ml diluted dye were added to 50 µl of tissue supernatant diluted 1:10 (v/v) with distilled water. The mixture was incubated at room temperature for 5 min, then the absorbance was measured at 700 nm using a UV-vis spectrophotometer.

CAT activity was determined using the method of Aebi and Bengmayer (1974) and H_2O_2 (initial concentration: 10 mM) rate constant k (dimension: s-1, k) was determined from the absorbance at 240 nm. CAT activity was measured using the method reported. Hydrogen peroxide (H_2O_2) absorbs ultraviolet light; the wavelength of maximum absorption is 240 nm. The decomposition of H_2O_2 into water and oxygen by catalase in the supernatant is measured by reduction of absorbance at 240 nm. The observed decrease in absorbance was recorded for 1 min to measure the activity of the enzyme.

For total oxidant status (TOS) measurement, a microplate reader (BioTek Synergy H1; BioTek Instruments, Inc., Winooski VT) was set to 25º C; 500 µl reagent 1 (measurement buffer) and 75 µl tissue supernatant were combined and the absorbance was measured at 530 nm according to the kit procedure (Rel Assay Diagnostics, Gaziantep, Turkey). Twenty-five microliters of reagent 2 (pro-chromogenic solution) was added to the mixture and the product was incubated 25° C for 10 min. After incubation, TOS levels were determined by measuring the absorbance at 530 nm (Erel 2005).

For total antioxidant status (TAS) measurement, a microplate reader, BioTek Synergy H1 (BioTek Instruments, Inc.) was set to 25° C, 500 µl reagent 1 (measurement buffer) and 30 µl tissue supernatant were combined and the absorbance measured at 660 nm. Reagent 2 (colored ABTS solution), 75 µl, was added to the mixture and incubated at 25° C for 10 min. TAS levels were determined by measuring the absorbance at 660 nm (Erel 2004).

Blood samples were centrifuged at 600 x g at 4° C for 20 min to obtain the plasma. Aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels were determined using kits and enzymatic-colorimetric method with an auto-analyzer (Architect C8000, Abbott Park, IL). AST (Architect/Aeroset Aspartate Aminotransferase Reagent Kit), ALT levels (Architect/Aeroset Alanine Aminotransferase Reagent Kit) and ALP levels (Architect/Aeroset Alkaline Phosphatase Reagent Kit), Fischer Scientific Co., Middletown, VA).

Preparation of tissues for histological analysis and assessment

Liver samples were prepared for light microscopy by fixing in 10% neutral buffered formaldehyde at room temperature for 48 h. Samples were dehydrated through an ascending series of ethanols, cleared in xylene and embedded in paraffin. Sections were cut at 6 µm and mounted on slides. Sections were dewaxed with xylene and rehydrated through descending ethanols to distilled water, stained with hematoxylin and eosin (H & E) for routine examination and Gomori's trichrome for connective tissue and fibrosis. (Bancroft and Gamble 2002). All sections were examined by a histologist, blinded to the identity of the specimens, using a light microscope (Eclipse Ni-U; Nikon Corp., Tokyo, Japan). Images were obtained using a camera (DS-Fi2) and analyzed with the Image Analysis System (NIS-Elements Documentation; Nikon).

Histopathologic damage including intracytoplasmic vacuolization in hepatocytes, sinusoidal dilation, infiltration of inflammatory cells, vascular congestion and thickening of Glisson's capsule were scored as: 0, normal; 1, mild; 2, moderate; 3, severe, and total damage score (TDS) was calculated as the total score of the five tissue damage parameters for each liver sample, with a maximum total score of 15.

Statistical analysis

We used IBM SPSS statistics for Windows, version 22.0. (IBM Corp., Armonk, NY) for statistical analysis. Normal distribution of the data was determined by Shapiro-Wilk test. The data were summarized as median, minimum and maximum values. The Kruskal-Wallis test was used for group comparisons and the Conover paired comparison method was

Table 1. Liver tissue oxidant-antioxidant parameters

Groups	MDA (nmol/g)	GSH (nmol/g)	SOD (U/g protein)	CAT (K/g protein)	TAS (mmol/l)	TOS (μmol/l)
C	715 (707 – 740)[a]	2024 (2002 – 2098)[a]	57 (53 – 62)[a]	6.7 (6.3 – 6.9)[a]	1.3 (0.9 – 1.6)[a]	35 (31 – 40)[a]
CO	719 (704 – 746)[a]	2032 (2012 – 2067)[a]	59 (52 – 65)[a]	6.6 (6.1 – 6.9)[a]	1.2 (0.9 – 1.5)[a]	36 (32 – 41)[a]
TCDD	1115 (1025 – 1200)[b]	1491 (1406 – 1564)[b]	26 (21 – 31)[b]	2.1 (1.8 – 2.4)[b]	0.3 (0.2 – 0.4)[b]	66 (63 – 72)[b]
TQ	504 (428 – 564)[c]	2637 (2481 – 2788)[c]	91 (84 – 99)[c]	12.4 (10.5 – 14.4)[c]	3.7 (3.3 – 3.9)[c]	25 (21 – 32)[c]
TCDD + TQ	667 (607 – 702)[d]	1975 (1881 – 1987)[d]	46 (44 – 51)[d]	5.5 (4.7 – 6.4)[d]	1 (0.8 – 1.2)[d]	36 (33 – 42)[a]

C, control; CO, corn oil; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TQ, tymoquinene. MDA, malondialdehyde; GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; TAS, total antioxidant status; TOS, total oxidant status. Data are medians (min–max) of ten animals. Different letters in columns are significantly different. Groups with different letters in columns are significantly different from each other ($p < 0.05$).

used. The significance level was accepted as $p \leq 0.05$ for all tests.

Results

We found that application of TCDD for 30 days significantly increased MDA, TOS, ALT, AST and ALP levels, and reduced GSH, TAS, SOD and CAT levels ($p < 0.05$) compared to all other groups. In the TQ group, we found that GSH, SOD, CAT and TAS levels were increased significantly compared to all other groups, and MDA, TOS, ALT, AST and ALP levels were decreased compared to all other groups ($p < 0.05$). We also found that in the TCDD + TQ group, MDA, TOS, ALT, AST and, ALP levels that had increased in the TCDD group approached control group levels, and that GSH, TAS, SOD, CAT levels that had decreased in the TCDD group increased and approached the C group levels ($p < 0.05$). We found no significant difference between the C and CO groups (Tables 1 and 2).

Liver sections in C (Fig. 1), CO (Fig. 2) and TQ (Fig. 3) groups exhibited normal histological appearance. TCDD administration caused thickening of Glisson's capsule, intracytoplasmic vacuolization in hepatocytes, sinusoidal dilation, vascular and sinusoidal congestion and inflammatory cell infiltration, especially in the periportal area (Fig. 4). The intracytoplasmic vacuolization in hepatocytes and sinusoidal dilation in the TCDD group were not observed in the TCDD + TQ group. Furthermore, thickening of Glisson's capsule and sinusoidal congestion in the TCDD group were reduced significantly compared to the TCDD + TQ group (Fig. 5, Table 3).

The TDS demonstrated that there were no significant differences among the C, CO and TQ groups. The TCDD group exhibited increased TDS compared to all other groups. In the TCDD + TQ group, a significant decrease in TDS was observed compared to the TCDD group ($p < 0.05$) (Table 3).

Discussion

Toxicity caused by environmental pollutants plays a role in several chronic diseases including cancer. It has been reported that oxidative stress plays an important role in long term TCDD toxicity (Hassoun et al. 2000, Alsharif et al. 1994). Although the mechanism by which oxidative stress due to exposure to TCDD is unknown, it has been reported that TCDD increases lipid oxidation, 8-hydroxy 2-deoxyguanosine (8-OHdG)

Table 2. Serum ALT, AST, ALP levels

Groups	ALT (IU/l)	AST (IU/l)	ALP (IU/l)
C	36 (34 – 39)[a]	95 (91 – 99)[a]	198 (193 – 212)[a]
CO	36 (32 – 39)[a,d]	94 (91 – 98)[a]	200 (192 – 205)[a]
TCDD	102 (98 – 105)[b]	152 (145 – 164)[b]	259 (254 – 267)[b]
TQ	26 (24 – 99)[c]	88 (84 – 159)[c]	190 (186 – 198)[c]
TCDD + TQ	34 (31 – 36)[d]	124 (119 – 128)[d]	209 (205 – 212)[d]

C, control; CO, corn oil; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TQ, tymoquinene. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase. Data are medians (min–max) of ten animals. Different letters in columns are significant. Groups with different letters in columns are significantly different from each ($p < 0.05$).

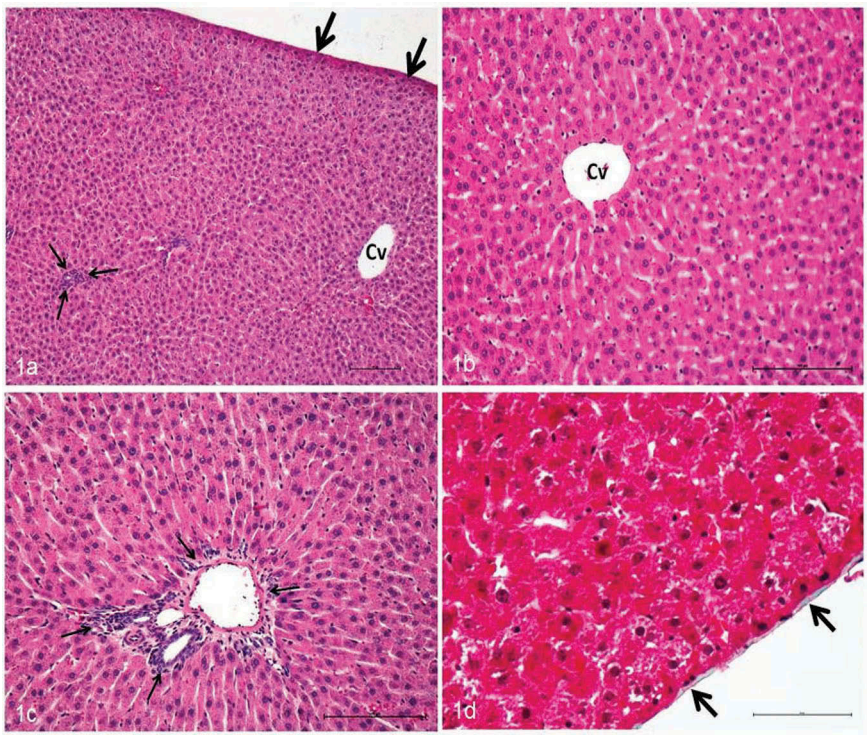

Fig. 1. Group C. a) Glisson's capsule (thick arrows), central vein (Cv), portal area (thin arrows). H & E. x 100. b) Central vein (Cv). H & E. x 200. c) Portal area (arrows). H & E. × 200. d) Glisson's capsule (arrows). Gomori's trichrome. x 400.

content and DNA damage and superoxide formation. TCDD decreases reduced glutathione (GSH) content, liver membrane fluidity and nonprotein sulfhydryl content (Alsharif and Hassoun 2004, Chan et al. 2004).

Antioxidants include any substance that delays or inhibits oxidative damage to a target molecule (Mochida et al. 2007). Animals possess various antioxidant defense systems under normal physiological conditions to prevent the formation of free radicals and therefore the damage that they can cause. The antioxidants in this system also participate in the removal of free radicals, which prevents oxidative damage (Urso and Clarkson 2003, Aydın et al. 2001).

It has been reported that TCDD decreased antioxidant enzyme activity and increased lipid oxidation, which led to oxidative stress in rats (Bentli et al. 2016, Ciftci et al. 2013, Turkez et al. 2012). Bentli et al. (2016) administered 2 µg/kg/week TCDD and 10 mg/kg/day montelukast (ML) as a protective agent to rats for 60 days and the liver tissue was examined. These investigators reported that TCDD significantly decreased GSH, CAT and

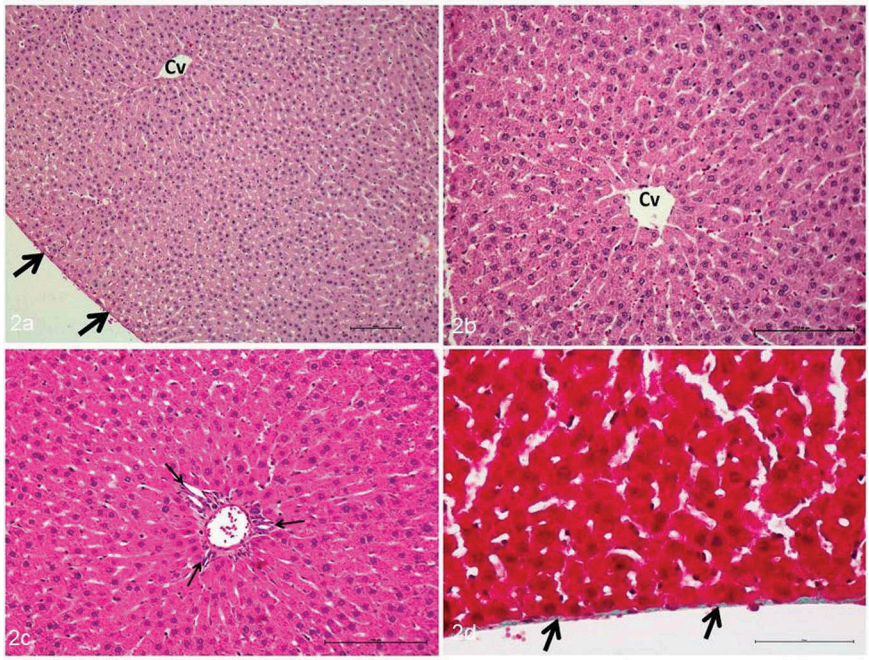

Fig. 2. Group CO. a) Glisson's capsule (arrows), central vein (Cv). H & E. × 100. b) Central vein (Cv). H & E. × 200. c) Portal area (arrows). H & E. × 200. d) Glisson's capsule (arrows). Gomori's trichrome. × 400.

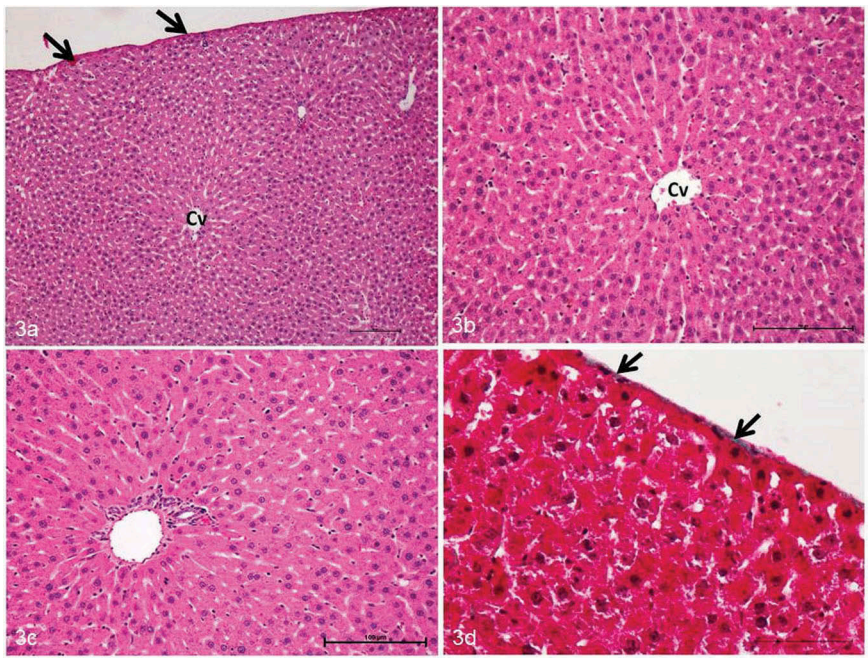

Fig. 3. Group TQ. a) Glisson's capsule (arrows), central vein (Cv). H & E. × 100. b) Central vein (Cv). H & E. × 200. c) Portal area (arrows). H & E. × 200. d) Glisson's capsule (arrows). Gomori's trichrome. × 400.

SOD levels, and caused mononuclear cell infiltration, intracytoplasmic vacuolization, congestion and mitotic hepatocytes, while ML caused significant improvement in oxidative stress parameters and histological findings compared to controls (Bentli et al. 2016).

Turkez et al. (2012) administered 50 µg/kg propolis as a preservative in an 8 µg/kg TCDD

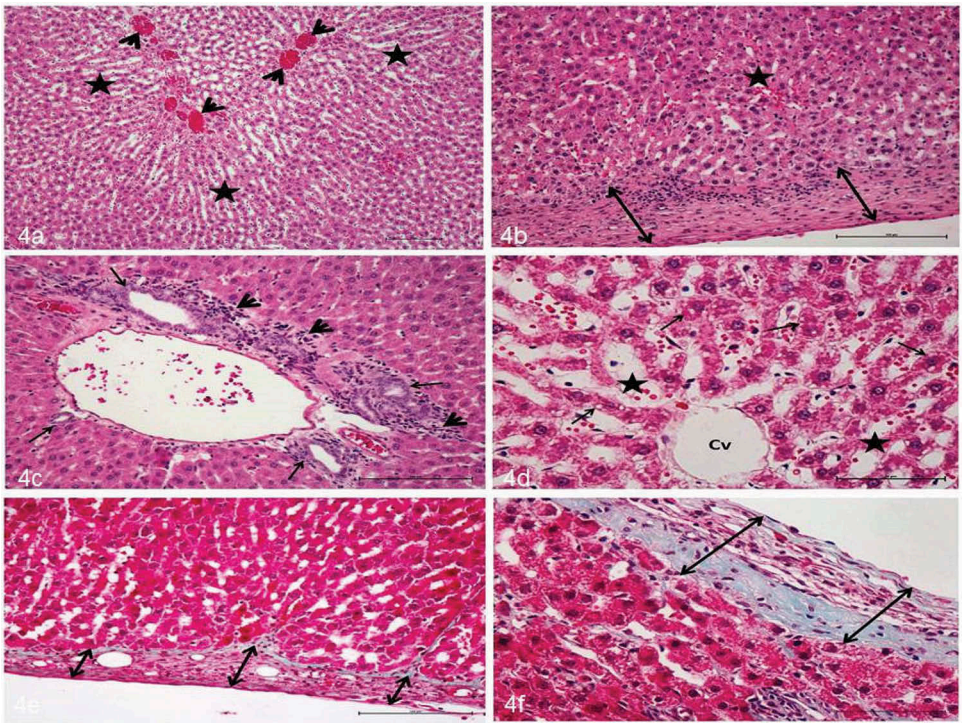

Fig. 4. Group TCDD. a) Sinusoidal dilation (*) and vascular congestion (arrowheads). H & E. × 100. b) Sinusoidal congestion (*), thickening of Glisson's capsule (bi-directional arrows), inflammatory cell infiltration in Glisson's capsule (arrowhead). H & E. × 200. c) Portal area (arrows) and inflammatory cell infiltration in periportal area (arrowheads). H & E. × 200. d) Central vein (Cv), intracytoplasmic vacuolization in hepatocytes (arrows), sinusoidal dilation (*). H & E. × 400. e) Thickened Glisson's capsule (bi-directional arrows). Gomori's trichrome. × 200. f) Thickened Glisson's capsule (bi-directional arrows). Gomori's trichrome. × 400.

induced rat hepatotoxicity model. These investigators reported that TCDD reduced SOD, CAT and GSH levels, and caused dilation of sinusoids, lymphocyte infiltration, parenchymal degeneration and lipid accumulation compared to controls. These investigators reported also that antioxidant parameters that had been reduced significantly after TCDD administration were increased to control group levels with TCDD + propolis administration and morphologic problems were ameliorated (Turkez et al. 2012).

Kalaiselvan et al. (2016) administered 100 ng/kg TCDD with 10 ml/kg olive oil (OO) as a preservative to male Wistar rats for 25 days. These investigators reported that TCDD increased ALT, AST, ALP, TBARS levels, decreased GSH and CAT levels and caused lymphocytic infiltration in liver tissue. TCDD + OO administration eliminated TCDD induced hepatotoxicity.

Bentli et al. (2013) administered 2 µg/kg/week TCDD and 50 mg/kg/day hesperidin (HP) to rats for 60 days and reported that TCDD increased TBARS levels, decreased GSH, SOD, CAT levels, increased necrotic cell formation, congestion, cell infiltration and vacuolization compared to controls; TCDD + HP application improved the adverse effects.

Ciftci et al. (2013) studied liver tissues in rats treated with 2 µg/kg/week TCDD with 50 mg/kg/day chrysin (CH) as a preservative and 20 mg/kg/day quercetin (Q). These investigators reported that TCDD increased significantly MDA levels, decreased SOD, CAT and GSH levels, increased vacuolization, eosinophilic cytoplasm and swelling. TCDD + Q and TCDD + CH groups decreased the increased MDA levels, increased the decreased SOD, CAT, GSH levels, and improved histopathological findings compared to the TCDD group (Ciftci et al. 2013).

Kimura et al. (2016) investigated the effects on fetal rat brain tissue of 0.6 and 3 µg/kg TCDD administered to pregnant rats between day 12.5 of gestation and birth. These investigators reported that TCDD caused anomalies in the brain and nerve tissues.

Sayed-Ahmed et al. (2010) administered 4 mg/kg TQ as a protective agent to rats in a 200 mg/kg diethyl-nitrosamine (DENA) induced hepatic carcinogenesis model. These investigators reported that

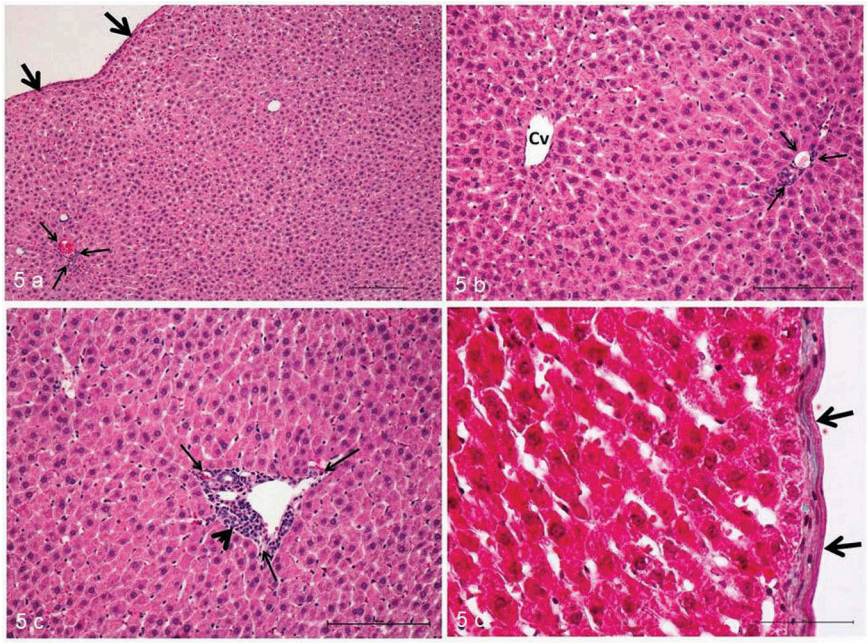

Fig. 5. Group TQ + TCDD. a) Glisson's capsule (thick arrows), portal area (thin arrows). H & E. × 100. b) Central vein (Cv), portal area (arrows). H & E. × 200. c) Central vein (Cv), portal area (arrows) and inflammatory cell infiltration in periportal area (arrowheads). H & E. × 200. d) Minimal thickening of Glisson's capsule (bi-directional arrows). Gomori's trichrome. × 400.

Table 3. Liver tissue histological damage scores

Groups	HDS
C	0 (0 – 0)[a]
CO	0 (0 – 0)[a]
TCDD	9 (7 – 10)[b]
TQ	0 (0 – 0)[a]
TCDD + TQ	3 (2 – 4)[c]

C, control; CO, corn oil; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TQ, tymoquinene. Data are medians (min–max) of ten animals. HDS, histological damage score. Different letters in columns are significant. Groups with different letters in columns are significantly different from each other. $p < 0.05$.

DENA increased serum ALT, ALP levels, and MDA levels in liver tissue, and decreased GSH and CAT levels. TQ administration decreased ALT, ALP and MDA levels and increased GSH and CAT levels.

Alhebshi et al. (2013) investigated an amyloid β induced Alzheimer's model in rats and reported that TQ, administered as a protective agent, inhibited reactive oxygen species and protected against neurotoxicity.

We investigated the association between TCDD and TQ using a TCDD hepatotoxicity model that altered antioxidant-oxidant parameters, histology and liver enzymes. Our findings concerning oxidant-antioxidant parameters, liver function enzyme tests and histopathology are consistent with those reported in the literature. TQ exhibited strong antioxidant properties and changed the oxidant/antioxidant balance in favor of antioxidants in the liver tissue. It was possible to decrease the effects of TCDD toxicity by TQ administration. TQ plays a preventive and protective role against TCDD hepatotoxicity.

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