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Does posttreatment thymoquinone reverse high-dose, atorvastatin-induced hepatic oxidative injury in rats?

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ABSTRACT

Atorvastatin (ATO) was commonly used to lower blood cholesterol, but it caused harmful effects to organs, including the liver. Thymoquinone (TQ), a prominent constituent of *Nigella sativa*, has antioxidant, antiinflammatory, antiapoptotic, antimicrobial, and anticancer activity. The current study investigated the mechanism of ATO-induced hepatotoxicity, whether posttreatment TQ could reverse ATO-induced hepatic injury, and the mechanism of action of TQ as a hepatoprotective agent. Forty adult male Sprague Dawley rats were divided into 4 equal groups: control, TQ-treated, ATO-treated, and combined ATO/TQ-treated. Rats were treated for 8 weeks and 10 days and euthanized by cervical dislocation 3 days after the last treatment. Blood samples and livers were tested for liver enzymes, oxidative stress, and apoptosis markers and for histopathological and ultrastructural examination. The ATO-treated group showed an increase in liver enzymes, decreases in reduced glutathione and catalase, and increases in the malondialdehyde lipid peroxidation marker, protein carbonylation, and caspase 3 activity. Posttreatment TQ in the ATO/TQ-treated group seemed to reverse these changes. Histopathological and ultrastructural examination supported these data. Results from the current study suggested posttreatment TQ may reverse oxidative stress injury in rat liver produced by ATO, suggesting a potential clinical application of using TQ to prevent ATO-induced hepatic injury.

Key words: atorvastatin, thymoquinone, oxidative stress, apoptosis, liver

INTRODUCTION

Atorvastatin (ATO) was a commonly drug used to lower blood cholesterol, but its use is currently limited because it caused harmful side effects to many organs, including the liver (Pal et al. 2015). ATO is a statin that decreases production of low-density lipoprotein cholesterol by blocking action of HMG-CoA reductase enzyme in the liver at the rate-limiting step of cholesterol biosynthesis (Jacobson 2008).

A primary cause of hepatic injury is oxidative stress via reactive oxygen species (ROS), which can directly or indirectly cause DNA or RNA damage, protein carbonylation, and lipid peroxidation. If not accounted for, the damage can lead to cell death and apoptosis (Lu et al. 2010). Therefore, antioxidants are important for protecting cells from oxidative stress. By accepting or donating electrons to ROS, antioxidants can convert ROS to less toxic molecules.

Nigella sativa is a natural antioxidant that has been used to treat a variety of diseases, such as asthma, diabetes, fever, and hypertension (Khader and Eckl 2014). Thymoquinone (TQ) is a prominent constituent of the volatile oil of *Nigella sativa* seeds (Mabrouk, 2017) and has been investigated for its pharmacological effects, including antioxidant, antiinflammatory, antiapoptotic, antimicrobial, and anticancer activity. TQ has also been shown to offer hepatorenal protection against methotrexate-induced toxicity in rats (El-Sheikh et al. 2015).

Despite the number of studies investigating the antioxidant, antiinflammatory properties of TQ against oxidative stress, there is no available information whether TQ can protect against ATO-induced hepatotoxicity. Thus, the objective of the current study was to investigate the mechanism of ATO-induced hepatotoxicity, whether posttreatment TQ could reverse the ATO-induced hepatic injury, and the mechanism of action of TQ as a hepatoprotective agent.

MATERIALS AND METHODS

Materials

ATO, TQ, catalase activity (for rat homogenized liver tissue samples), and malondialdehyde (MDA) were purchased from Sigma-Aldrich Corp. (Saint Louis, MO, USA). OxiSelect catalase activity (for rat serum samples) was purchased from Cell Biolabs, Inc. (San Diego, CA, USA). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits were purchased from DIALAB Diagnostics Co. (Wiener Neudorf, Austria). The reduced glutathione colorimetric assay kit was purchased from Biodiagnostic (Cairo, Egypt). The protein carbonyl colorimetric assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). Caspase 3 assay kit (colorimetric) was purchased from Abcam (Cambridge, United Kingdom). Assays were analyzed using a spectrophotometer (DTN 402, DIALAB GmbH, Austria) at different wavelengths.

Experimental Design

Forty healthy, adult male Sprague Dawley rats (150-160 g) were purchased from the Animal House at the Faculty of Medicine at Cairo University in Cairo, Egypt. Rats were housed in a standard animal facility, which was maintained at a constant 23°C and 12:12-hour light-dark cycles. Rats had access to laboratory chow and water at all times. All persons involved in the study had completed animal care use and training. Experimental procedures, animal handling, sampling, and scarification followed the Guide for the Care and Use of Laboratory Animals, 8th edition (National Research Council 2011) and were approved by ethical committee of the Anatomy Department, Faculty of Medicine, Cairo University.

Before the start of the study, rats were left for 2 weeks to acclimatize. After that time, they were weighed and randomly divided into 4 groups of 10 rats. Group 1 (control group)

received only water for 8 weeks through gavage once daily. Group 2 (TQ-treated group) received water for 8 weeks through gavage once daily and then 100 mg/kg/day body weight of TQ orally (Mansour et al. 2002) for 10 consecutive days (El-Sheikh et al. 2015). Group 3 (ATO-treated group) received 30 mg/kg/day body weight of ATO for 8 weeks through gavage once daily (Pal et al. 2015). Group 4 (combined ATO/TQ-treated group) received 30 mg/kg/day body weight of ATO for 8 weeks through gavage once daily (Pal et al. 2015) and then ATO administration was discontinued and replaced by 100 mg/kg/day body weight TQ orally once a day (Mansour et al. 2002) for 10 consecutive days (El-Sheikh et al. 2015). Blood samples and livers from all groups were tested for liver enzymes, oxidative stress, and apoptosis markers and for histopathological and ultrastructural examination.

Sample Preparation

Three days after the last treatment of TQ, rats from all 4 groups were euthanized by cervical dislocation. Blood samples were collected from their caudal vena cava and centrifuged at 5000 rpm for 15 minutes. Serum was separated and stored at -80°C until use. Rat livers were dissected and weighed. Liver sections were placed in normal saline, processed in formalin (10%), and embedded in paraffin for morphological and cellular studies by histopathological examination. A series of 1 mm³ pieces of rat livers were fixed in glutaraldehyde (3%) for 2 hours at room temperature, washed in phosphate buffer (0.1 M, pH 7.4) at 4°C, and post fixed with osmium tetroxide (2%) at 4°C for 2 hours. Samples were dehydrated, embedded in Epon-Araldite at 1:1 for 1 hour, and polymerized in an oven for 24 hours at 60°C. The remaining liver tissues were kept at -80°C after snap freezing in liquid nitrogen. Liver tissue homogenate was prepared by homogenizing the liver samples in potassium phosphate buffer (0.01 M, pH 7.4). The liver homogenate was then centrifuged for 20 minutes at 3000 rpm. Aliquots (0.3 mL) of the

supernatant were collected and kept at -80°C until use to avoid repeated thawing and refreezing.

Assessment of Total Body Weight and Liver/Total Weight Ratio

To assess the effects of TQ alone, ATO alone, and combined ATO and TQ on body weight, rat body weight was recorded before the study and after the study just before the rats were euthanized. Rat livers were weighed after dissection, and the liver weight/body weight ratio was calculated.

Assessment of Liver Biomarkers in Rat Serum

Hepatocellular function and hepatocellular toxicity were assessed using standard colorimetric ALT and AST assay kits and the modified International Federation of Clinical Chemistry UV kinetic method. Assays were analyzed using a wavelength of 340 nm. Calibrations, quality controls, and assays were performed per manufacturer instructions using one calibration lot and one reagent lot for each parameter. Results were expressed in IU/L.

Assessment of ROS and Oxidative-Stress Markers in Rat Serum and Liver Homogenate

Calibrations, quality controls, and assays for ROS and oxidative stress markers were performed per manufacturer instructions using one calibration lot and one reagent lot for each parameter.

Reduced Glutathione Assay in Rat Serum and Liver Homogenate.—Reduced glutathione (GSH) is an important oxidative stress marker because it is an antioxidant that prevents the damage of cellular components by peroxides and free radicals of ROS (El-Sheikh et al. 2015). GSH was measured in rat serum and homogenized liver tissue, using a reduced glutathione colorimetric assay kit. Assays were analyzed using a wavelength of 405 nm. Results were expressed in $\mu\text{mol/L}$ in rat serum and $\mu\text{mol GSH/g}$ in liver tissue homogenate.

Catalase Assay in Rat Serum and Liver Homogenate.—Catalase is another oxidative

stress marker that was measured in rat serum and homogenized liver tissue, using a catalase activity colorimetric assay kit for both samples. Assays were analyzed using a wavelength of 520 nm. Results were expressed in U/mL in rat serum and U/g in liver tissue homogenate.

MDA Assay in Rat Serum and Liver Homogenate.—MDA is a marker for lipid peroxidation that was measured in rat serum and homogenized liver tissue, using a lipid peroxidation (MDA) colorimetric assay kit. Assays were analyzed using a wavelength of 532 nm. Results were expressed in nmol MDA/L in rat serum and nmol MDA/g in liver tissue homogenate.

Protein Carbonyl Assay in Rat Serum and Liver Homogenate.—Protein carbonylation is another marker of oxidative stress that was measured in rat serum and homogenized liver tissue, using a protein carbonyl colorimetric assay kit. Assays were analyzed using a wavelength of 385 nm. Results were expressed in nmol/mL in rat serum and nmol/mg in liver tissue homogenate.

Assessment of Apoptosis in Rat Liver Homogenate

Caspases (cysteine-requiring aspartate protease) are proteases that facilitate cell death. Caspase 3 activity was measured using a caspase 3 colorimetric assay kit. Assays were analyzed using a wavelength of 405 nm. Calibrations, quality controls, and assays were performed per manufacturer instructions, using one calibration lot and one reagent lot for each parameter. Results were expressed in arbitrary units.

Assessment of Liver Histology by Light Microscopy

Paraffin blocks of the liver tissues were sectioned at 5 μ m thickness, stained with hematoxylin and eosin (H&E) and Masson's trichrome stain, and examined for histopathology using Leica Qwin 500 LTD light microscope (Cambridge, UK).

Ultrastructural Assessment of Liver by Electron Microscopy

Semithin sections of rat livers, 1 μm thick, were fixed on copper grids, stained with toluidine blue (1% solution), and examined under a light microscope. Ultrathin sections of rat livers, 50-90 nm thick, were examined using a JEOL 100CX TEM (Hertfordshire, UK) transmission electron microscope.

Statistical Analysis

SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) was used to perform all analyses. Shapiro-Wilk tests for normality and Brown-Forsythe tests for equal variance were used to select appropriate statistics and tests. Descriptive statistics were summarized for each group, using means and standard deviations (SD) for normal data, and medians and interquartile ranges (IQR) for non-normal data. To determine differences between groups, analysis of variance was used for normal data and Kruskal-Wallis tests for non-normal data. The type I error rate in pairwise comparisons was controlled using Tukey's method for significant analysis of variance results or Dunn's method for significant Kruskal-Wallis results. The significance level was set at $P < .05$.

RESULTS

Significant differences were found between groups for all outcome measures (all $P < .001$) (Table 1, Figure 1). Differences were found between Groups 1 and 2 for the liver weight/total body weight ratio, GSH serum, and MDA homogenized liver tissue.

Differences were found between Groups 1 and 3 for all outcome measures (all $P < .001$), except catalase serum (Table 1, Figure 1). Outcomes for Group 3 were greater than Group 1 for liver weight/total body weight ratio, ALT serum, AST serum, MDA serum, MDA homogenized liver tissue, protein carbonylation serum, protein carbonylation homogenized liver tissue, and

caspase 3 homogenized liver tissue and were less than Group 1 for the total body weight, GSH serum, GSH homogenized liver tissue, and catalase homogenized liver tissue.

Differences between Groups 1 and 4 were found for several measures (all $P < .001$) (Table 1, Figure 1). Outcomes for Group 4 were greater than Group 1 for AST serum, catalase serum, MDA homogenized liver tissue, protein carbonylation serum, protein carbonylation homogenized liver tissue, and caspase 3 homogenized liver tissue and were less than Group 1 for GSH homogenized liver tissue.

When comparing treated groups (Groups 2-4), differences were found between the 3 groups for AST serum (Group 2 < Group 4 < Group 3), GSH homogenized liver (Group 3 < Group 4 < Group 2), and MDA homogenized liver (Group 2 < Group 4 < Group 3) (Table 1). Group 2 was different from Groups 3 and 4 for GSH serum (Group 2 > Groups 3, 4), catalase homogenized liver (Group 2 > Groups 3, 4), and protein carbonylation homogenized liver (Group 2 < Groups 3, 4). Group 3 was different from Groups 2 and 4 for protein carbonylation serum (Group 3 > Groups 2, 4). Groups 2 and 3 were the only treated groups that had differences for total body weight (Group 3 < Group 2), ALT serum (Group 2 < Group 3), MDA serum (Group 2 < Group 3), and caspase 3 homogenized liver (Group 2 < Group 3). Groups 2 and 4 were the only treated groups that had differences for liver weight/total body weight ratio (Group 4 < Group 2). Groups 3 and 4 were the only treated groups that had differences for catalase serum (Group 3 < Group 4).

Assessment of Liver Histology by Light Microscopy

Group 1 H&E-stained sections had normal architecture of the hepatic lobules and were composed of central veins and masses of liver cells (hepatocytes) with liver plates radiating from the central veins (Figure 2a). The hepatocytes were polyhedral cells with acidophilic cytoplasm.

Their nuclei were large, rounded, and vesicular and were nearly the same size. The liver plates were separated from each other by irregular blood sinusoids.

Group 2 H&E-stained sections had normal central vein and hepatic sinusoids similar to that of Group 1 (Figure 2b).

Group 3 H&E-stained sections had the central vein packed with red blood cells (RBCs), fluid exudate (Figure 2c), and lymph (Figure 2d), and there were dilated (Figures 2c and 2d), congested (Figure 2c) sinusoids compressing the surrounding hepatic cell plates. The cytoplasm had inflammatory mononuclear cellular infiltrate, vacuolations, and apoptotic cells with disfigured nucleus (Figure 2c).

Group 4 H&E-stained sections had congested central veins and dilated hepatic sinusoids, both of which were packed with RBCs (Figure 2e). The cytoplasm had multiple Kupffer cells, which lined the walls of the sinusoids, and binucleated cells with cytoplasmic vacuoles of variable sizes. Some of the nuclei of the binucleated cells were indented by the cytoplasmic vacuoles (Figure 2f).

In Masson's trichrome-stained sections, Groups 1 and 2 had minimal connective tissue around the central vein with negligible extension between the surrounding hepatocytes (Figures 3a and 3b, respectively). Group 3 had an increased amount of connective tissue around the central vein with dense collagen fibers extending between the surrounding hepatocytes (Figure 3c). Group 4 had a decreased amount of connective tissue around the central vein compared to Group 3, almost equal to the amount of connective tissue around the central vein seen in Group 1, with negligible extension between the surrounding hepatocytes (Figure 3d).

Ultrastructural Assessment of Liver Tissue by Electron Microscopy

Electron microscopic examination of liver sections of Group 1 showed normal

ultrastructural features of the hepatocytes (Figure 4a). The most conspicuous cytoplasmic organelles were the rounded or elongated mitochondria, which were defined by 2 limiting membranes and comprised of relatively dense mitochondrial matrix with mitochondrial cristae. The mitochondria were surrounded by an abundant amount of rough endoplasmic reticulum, which appeared as parallel arrays of fine tubules on the side of the nucleus. The outer surfaces of the rough endoplasmic reticulum cisternae were studded with ribosomes. The nucleus of the polygonal hepatocytes was rounded or oval with finely dispersed chromatin. Group 2 results were similar with normal architecture of the hepatocytes (Figure 4b).

Group 3 had alteration in the cytoplasmic organelles and nuclei of the hepatocytes. An increased number of mitochondria were detected in the cytoplasm of the hepatocytes (Figures 4c, 4d, and 4e). Some of the mitochondria were enlarged (Figures 4c and 4d), while others varied in shape and size (Figure 4e). The rough endoplasmic reticulum showed fragmentation and partial loss of ribosomes (Figures 4c and 4d). The ribosomes were scattered in the degenerated cytoplasm (Figure 4c). Excess, dense lysosomal bodies were in the cytoplasm of the hepatocytes (Figure 4c). The most striking feature in the cytoplasm of the hepatocytes was the presence of many fat droplets, which were not surrounded by membranes and were of variable sizes (Figure 4d). Some areas of the cytoplasm appeared vacuolated and rarified (Figure 4e). Condensed chromatin granules were in the hyperchromatic nuclei of the hepatocytes with irregular membrane (Figure 4d). The cytoplasmic organelles in Group 4 showed restoration of the structure of the cytoplasmic organelles and nuclei (Figure 4f).

DISCUSSION

The liver is the main site that delivers ATO for the HMG-CoA reductase enzyme, which

is a core enzyme in cholesterol biosynthesis (Jacobson 2008; Pal et al. 2015; Sliskovic et al. 1992). ATO is commonly well endured with minimal side effects, however, ATO-induced hepatocellular injury has been reported in 1.9%- 5.5% of patients with drug-induced hepatocellular injury following dose escalation of ATO. Mortality due to ATO-induced hepatocellular injury has been reported in few patients, while most patients have improved after termination of ATO therapy. (Björnsson, 2016). Further, research has shown that ATO-induced oxidative stress occurs through ROS (Qi et al. 2013). Examples of ROS include superoxide anion, hydrogen peroxide, and hydroxyl radical, which are chemically reactive molecules or free radicals that contain oxygen in their molecular structure (Ray et al. 2012). Thus, ATO-induced hepatotoxicity results from macromolecular and mitochondrial damage from increased intracellular production of ROS exceeding its natural antioxidant defense capacity (Halliwell and Gutteridge 2007).

In the current study, study groups were tested for liver enzymes, oxidative stress, and apoptosis markers and for histopathological and ultrastructural examination. No differences were found between Groups 1 and 2, which were the control and TQ-treated groups. However, statistically significant differences were found between Group 3, the ATO-treated group, and Groups 1 and 2 for all outcomes measures, except for catalase serum. There were also statistically significant differences between Group 3 and Group 4, the combined ATO/TQ-treated group.

In the current study, Group 3 had a lower total body weight and had a higher liver weight/total body weight ratio compared to Group 1. This result was explained in the histological analysis, which showed congestion in the central vein and sinusoids for Group 3 that were filled with RBCs, fluid exudate, and lymph.

The hepatotoxicity of Group 3 was evidenced by an increase in ALT serum and AST serum, which are membrane-leaking enzymes. There was also a simultaneous increase in protein carbonylation serum and MDA serum, which are markers of hepatic oxidative injury.

Glutathione is a vital antioxidant that prevents breakdown of the chief cellular components by ROS. Because of its oxidative breakdown of the lipid content of cell membranes, MDA is a marker for lipid peroxidation, and it acts as a marker of oxidative stress (Pal et al. 2015).

Results of the current study suggested the mechanisms involved in the ATO-induced hepatotoxicity seen in Group 3 were related to oxidative stress. We found simultaneous decreases in GSH serum, GSH homogenized liver tissue, and catalase homogenized liver tissue in Group 3. Similar findings related to ATO-induced hepatotoxicity were reported by Pal et al. (2015). The authors (Pal et al. 2015) also suggested that the ATO-induced hepatotoxicity reduced GSH-regenerating enzyme activity and that GSH scavenged ROS, transforming into an oxidized form. In another study, El-Sheikh et al. (2015) reported a decrease in GSH and catalase and an increase in MDA in response to ROS-induced injury by methotrexate in rat liver. In the current study, the reduction in the activity of GSH and catalase in Group 3 may be explained by ATO binding to glutathione reductase and catalase, respectively, and reducing the free enzymes available.

In the current study, the significant difference between Group 3 and Group 1 for all compared outcome measures (except catalase serum) and the differences between Group 3, on one side, and Groups 2 and 4, for all compared outcome measures were supported by histological analysis of Group 3, which showed dilated and congested central veins and sinusoids. Hepatocytes had degenerated cytoplasm, infiltrated by inflammatory cells, and disfigured nuclei. The vacuolated cytoplasm showed fatty degeneration of the liver tissue. Further, the increased

connective tissue around the central vein, shown in the Masson's trichrome histological sections of Group 3, indicated the beginning of hepatic fibrosis. These differences were also supported by ultrastructural analysis of Group 3, which showed alteration of the normal cytoplasmic organelles and nuclei, suggesting degeneration in the hepatocytes.

Previous research has suggested that the normal dose of ATO for rats below which hepatic injury does not occur is 20 mg/kg/day (Ekstrand et al. 2015). The current study was designed so that the 30 mg/kg/day body weight dose of ATO guaranteed hepatic injury (Pal et al. 2015). As expected, our results indicated that administering this high dose of ATO during the 8 weeks of our study caused oxidative stress and hepatic tissue damage in our rat model. It is likely that the administration of ATO depleted the antioxidant enzyme activity in rat hepatocytes, causing oxidative stress and resulting in ATO-induced hepatotoxicity.

ATO-induced oxidative stress in the rat hepatocytes could have a major impact on determining the formation of Mallory-Denk bodies (MDBs), in-vivo. MDBs are protein aggregates consisting mainly of K8 and K18 variants of the cytoplasm of the intermediate filaments (IFs) inside the hepatocytes (Strnad et., 2008). IFs are mainly responsible for supporting the cytoskeleton of the cytoplasm in the hepatocytes. They also arrange complex platforms and interact with various apoptotic proteins and kinases. The K8 and K18 variants in humans, influence the progress of acute liver failure and end-stage liver disease. These variants are also linked to the progress of hepatic fibrosis complicating chronic hepatitis C (Strnad et al., 2008). Thus, ATO-induced oxidative stress, by disrupting the formation of the MDBs in the hepatocytes, they could have indirectly interfered with the K8 and K18 cytoplasmic variants within the IFs, causing distortion in the shape of the hepatocytes and their intracellular organization.

This hypothesis was supported by Tarantino, et al., (2007) who reported that the K8 and K18 cytoplasmic variants of the IFs were key factors providing mechanical stability to the structure of the hepatocytes and represented a target for drug-induced oxidative stress in the hepatocytes, ultimately causing apoptosis/necrosis. Tarantino, et al., (2007) identified a serological reflection of K18, the tissue polypeptide-specific antigen (TPS), which was commonly used as a marker for several cancers. Tarantino, et al., (2007) added that TPS was a better marker than ALT, and/or ultrasonography for diagnosing non-alcoholic steatohepatitis (NASH) and differentiating it from fatty liver (FL).

Non-alcoholic fatty degeneration of the liver tissue was commonly coupled by apoptosis of the hepatocytes which was a characteristic feature of severe NASH and FL (Tarantino, et al., 2011). Apoptosis of the hepatocytes due to the exposure to free fatty acids, could increase the production of ROS with further mitochondrial damage. This devastating effect on the hepatocytes could be balanced by the administration of antioxidant materials (Tarantino, et al., 2011).

In Group 4, ATO administration was discontinued after 8 weeks and replaced by posttreatment TQ at a dose of 100 mg/kg/day body weight for 10 successive days to test whether TQ could alleviate ATO-induced hepatic tissue injury. In a previous study, a 10-day treatment of TQ resulted in protective effects against methotrexate-induced hepatorenal toxicity in rats and positive histological changes in the tissues (El-Sheikh et al. 2015). Badary et al. (1998) also reported that oral administration of TQ at a dose of 30, 60, and 90 mg/kg/day body weight did not alter hepatic GSH content. Mansour et al. (2002) studied TQ at a dose similar to that used in the current study and reported that the high dose of TQ reduced hepatic lipid peroxidation, but smaller doses (25 and 50 mg/kg body weight) did not to restore GSH content in the studied

tissues. A study by Al-Ali et al. (2008) suggested the use of TQ, especially when given orally, to treat methotrexate-induced toxicity in rats since TQ has shown to improve liver functions and produce positive histological changes in rat liver sections.

Posttreatment TQ in Group 4 of the current study resulted in significant antioxidant effect in rat livers. The total body weight increased and the liver weight/total body weight ratio was decreased compared to Group 3. This finding was supported by reductions in the amount of connective tissue around the central vein found in the Masson's trichrome histological sections of Group 4, which were different from those of Groups 1 and 3 where the amount of collagen fibers was less in Group 4 compared to Group 3, with minimal fibrosis between the surrounding hepatocytes. These findings suggested that posttreatment TQ noticeably reduced the amount of ATO-induced hepatic fibrosis. TQ has been proven to protect against lead (Pb)-induced hepatocellular injury in rat via its antioxidant properties which could have a potential clinical application of using TQ to prevent Pb-induced hepatocellular injury in humans. (Mabrouk, 2017). TQ also protected against ischemic/reperfusion hepatocellular injury in rat via preventing mitochondrial dysfunction and endoplasmic reticulum stress thus ameliorating oxidative stress (Bouhel et al., 2017).

Another method to reduce the amount of drug-induced hepatic fibrosis in animal model was proposed by Molina-Aguilar, et al., (2017) using time-caloric restriction protocol. This protocol basically allowed only two hours of access to food during the daytime for 18 weeks and was found to preserve the histological architecture and functional characteristics of the fibrotic areas in the liver via enhancing collagen deposition and preventing systemic inflammation.

In Group 4, the significant reductions in ALT serum and AST serum and the increases in GSH, catalase in serum, and homogenized liver tissue reflect the antioxidant properties of TQ.

These antioxidant properties were supported by significant reductions in lipid peroxidase as measured by MDA and protein carbonylation in serum and homogenized liver tissue. The restoration of GSH and catalase after posttreatment TQ may be explained by upregulation of gene expression caused by TQ rather than protein synthesis. Further, TQ may have induced DNA synthesis, producing more glutathione reductase and catalase. TQ inhibited the inflammatory reaction and the process of oxidative stress, possibly by decreasing the levels of pro-inflammatory cytokines, altering the membrane-bound enzyme activity and Phases I and II antioxidant enzymes. This proposed mechanism of action ultimately inhibited the expression of Nuclear factor kappa beta, which could have a potential clinical application of using TQ to inhibit the growth of hepatocellular carcinoma (Verma et al., 2017). Additional studies are needed to explain the exact mechanisms by which posttreatment TQ increases the activity of antioxidant enzymes.

Similar findings for GSH, catalase, and MDA were reported by El-Sheikh et al. (2015) after the administration of TQ to treat ROS induced by methotrexate in rat liver. However, Dal-Pizzol et al. (2001) reported that pretreatment with ROS scavengers decreased the elevated catalase and MDA activity. In other studies, Nagi and Mansour (2000) and Mansour et al. (2002) reported decreases in catalase and MDA after oral administration of TQ. Mansour et al. (2002) suggested the decrease in catalase and MDA were caused by less availability of free radicals, which are substrates for these enzymes, because of the free radical scavenging effect of TQ.

In the current study, the findings in Group 4 were supported by histological analysis, which revealed congested central veins and dilated sinusoids. Hepatocytes had degenerated cytoplasm, infiltrated by Kupffer cells, and cytoplasmic vacuolations indenting the nuclei. Roberts et al. (2007) suggested Kupffer cells have a role in acute and chronic responses to

hepatotoxicity, causing the release of inflammatory mediators, growth factors, and ROS. Thus, the TQ-induced release of Kupffer cells may be attributed to antiinflammatory properties of TQ, which counteract ATO-induced hepatotoxicity. The decreased amount of connective tissue around the central vein in Group 4 suggested that TQ was effective at reversing hepatic fibrosis. This conclusion was also supported by our ultrastructural analysis of Group 4, which showed restoration of the structure of the cytoplasmic organelles and nuclei. Taken together, these results suggested that posttreatment TQ alleviated the ATO-induced hepatotoxic effect in rat liver.

In the current study, there was a significant difference between Groups 3 and 1 for caspase 3 homogenized liver tissue. This result was confirmed during histological analysis of Group 3 because apoptotic cells were observed in the rat liver tissues. The mechanism of caspase 3 activation induced by ATO observed in the current study was explained by Gao et al. (2003) as an intracellular programmed cell death mechanism that allows injured cells affected by oxidative stress to self-destroy through reduction in the membrane potential of the mitochondria. This causes the release of cytochrome C, apoptosome formation, and activation of the caspase cascade and other pro-apoptotic proteins in the cytosol (Gao et al. 2003). Thus, caspases are widely considered as the executors of the apoptotic mechanism (Hengartner 2000 and Tarantino, et al., 2011). Increased blood levels of cytochrome C, triglycerides, gamma-glutamyl transferase, and unconjugated bilirubin were detected in patients with NASH and FL (Tarantino, et al., 2011).

In the current study, Group 4 findings for caspase 3 homogenized liver tissue were greater than those of Group 1, suggesting posttreatment TQ prevented the creation of apoptotic bodies and protected the rat liver through this antioxidant property. This result was supported by previous studies where antiapoptotic properties of TQ in liver were shown to decrease expression of caspase 3 as a marker of apoptosis (El-Sheikh et al. 2015; Helal 2010). However, the

anticancer properties of TQ have recently been investigated in vivo and in vitro with certain types of malignancies, and TQ was found to induce the expression of caspase 3, thus promoting apoptosis (Attoub et al. 2013).

To our knowledge, the current study is the first to demonstrate a posttreatment protective effect of TQ against high-dose, ATO in rat liver. Further, our results may be a valuable contribution to the existing literature, particularly since TQ is currently being investigated with a wide range of compounds because of its antioxidant, antiinflammatory (El-Sheikh et al. 2015; Mansour et al. 2002), and antiapoptotic (Helal 2010) properties and its anticancer activities against certain tumors (Attoub et al. 2013).

The current study had some limitations. Although results of the current study proposed an effect of posttreatment TQ against high dose, ATO-induced hepatotoxicity, the study used a rat model, and the assumption that the same effects of TQ can be replicated with human patients cannot be made. Future studies should evaluate the effect of TQ to prevent high-dose, ATO-induced hepatotoxicity in a rat model and compare the efficiency of administering TQ before high-dose ATO and concomitantly to determine whether the structural histological damage caused by high-dose ATO can be reversed or not. Future studies should also investigate the posttreatment use of TQ to ameliorate high-dose, ATO-induced hepatotoxicity in humans. A study could also assess the effect of different doses of TQ to determine whether increasing the dosage increases the difference between Groups 3 and 4.

CONCLUSION

Results of the current study suggested posttreatment TQ may protect against high-dose, ATO-induced oxidative damage in the rat liver. These results may be a key factor for

investigating the protective effect of TQ against a variety of other oxidative stress-producing toxic substances. Further, our data may contribute to the understanding of the endogenous antioxidant, antiinflammatory, and antiapoptotic properties of TQ and may eventually explain the possible mechanisms of action of TQ. These observations suggest that TQ may be a clinically valuable agent to prevent high-dose, ATO-induced hepatocellular damage caused by oxidative stress, such as in conditions of hypercholesterolemia that are treated with high doses of ATO.

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FIGURE LEGENDS

Figure 1. Violin plots of outcome measures for each study group.

Figure 2. Histological examination of hematoxylin and eosin-stained sections of rat liver for all study groups. **a.** Group 1 (control) showing normal architecture. Note the central vein (C) and hepatic sinusoids (S). **b.** Group 2 (TQ-treated group) showing normal central vein (C) and hepatic sinusoids (S) similar to that of Group 1. **c.** Group 3 (ATO-treated group) showing the central vein (C) packed with red blood cells and fluid exudate (arrowheads) and dilated congested sinusoids (S) compressing the surrounding hepatic cell plates. Note the vacuolated cytoplasm (arrows) and the apoptotic cell (P) with disfigured nucleus and increased cytoplasmic inflammatory mononuclear cells (circles). **d.** Group 3 showing lymph congestion in the central vein (C). Note the dilated sinusoids (S). **e.** Group 4 (combined ATO/TQ-treated group) showing congested central veins (C) and dilated hepatic sinusoids (arrows); both appear packed with red blood cells. **f.** Group 4 showing binucleated cells (B) with cytoplasmic vacuoles of variable sizes, one of which was indenting the nucleus (arrowhead). Note the multiple Kupffer cells around the sinusoids (arrows).

Figure 3. Histological examination of Masson's trichrome-stained sections of rat liver for all study groups. **a.** Group 1 (control) showing minimal connective tissue (arrows) around the

central vein (C). There is negligible extension between the surrounding hepatocytes. Note the hepatic sinusoid (S). **b.** Group 2 (TQ-treated group) showing minimal connective tissue area (arrows) in the hepatic lobule in one field. There is negligible extension between the surrounding hepatocytes. **c.** Group 3 (ATO-treated group) showing an increase in the amount of connective tissue (arrows) around the central vein (C) and extending between the surrounding hepatocytes (circles). **d.** Group 4 (combined ATO/TQ-treated group) showing a decrease in the amount of connective tissue (arrow) around the central veins (C) of the hepatic lobules compared to Group 3. There is negligible extension between the surrounding hepatocytes.

Figure 4. Ultrastructural examination of rat liver for all study groups. **a.** Group 1 (control) showing regularly shaped nucleus (N) with finely dispersed chromatin. The rounded or oval mitochondria (M) are defined by 2 limiting membranes and comprised of relatively dense mitochondrial matrix with mitochondrial cristae. The rough endoplasmic reticulum (rER) appears as parallel arrays of fine tubules situated in the cytoplasm on the side of the nucleus. **b.** Group 2 (TQ-treated group) showing normal euchromatic nucleus (N) with an average number of oval and rounded mitochondria (M) and a few lysosomes (L). **c.** Group 3 (ATO-treated group) showing numerous enlarged mitochondria (M), discrete fragments of rough endoplasmic reticulum (arrows) with scattered ribosomes (circles), and excess lysosomes (L). Note the degenerated mitochondrion (D) with loss of mitochondrial matrix. **d.** Group 3 showing numerous enlarged mitochondria (M), fat droplets (F), and a hepatic nucleus (N) with condensed chromatin and irregular nuclear membrane. Note the discrete fragments of rough endoplasmic reticulum (arrows) **e.** Group 3 showing numerous mitochondria around the nucleus (N), of different shapes and sizes (M), increased number of lysosomes (L), and vacuolated (V), rarified cytoplasm (arrow). **f.** Group 4 (combined ATO/TQ-treated group) showing restoration in the structure of

the cytoplasmic organelles and nuclei; normal nucleus (N), numerous rounded and oval mitochondria (M), and normal rough endoplasmic reticulum (rER) are arranged in parallel tubules to the side of the nucleus.

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Table 1. Results for the outcome measures for all study groups.

Outcome Measure	Units	Study Group				<i>P</i> Value [†]
		Group 1	Group 2	Group 3	Group 4	
Total body weight	gm	155.5 (152, 157) ^b	155.5 (152, 157) ^d	147.5 (141, 149)	152.5 (148, 155)	<.001
Liver weight/total body weight ratio	NA	0.005 (0.005, 0.005) ^{a,b}	0.05 (0.05, 0.05) ^e	0.0135 (0.012, 0.015)	0.0105 (0.009, 0.012)	<.001
ALT serum	IU/L	18 (16, 20) ^b	17.5 (16, 18) ^d	51.5 (46, 60)	20.5 (19, 22)	<.001
AST serum	IU/L	18.5 (3.84) ^{b,c}	17.6 (2.17) ^{d,e}	53.5 (8.11) ^f	22.8 (2.57)	<.001
GSH serum	μmol/L	27 (26, 27) ^{a,b}	28 (27, 28) ^e	18 (16, 19)	25 (24, 25)	<.001
GSH homogenized liver	μmol	23.5 (1.35) ^{b,c}	23.4 (0.97) ^{d,e}	12.7 (1.16) ^f	18.2 (0.79)	<.001
Catalase serum	U/mL	0.5 (0.4, 0.5) ^c	0.5 (0.5, 0.6)	0.2 (0.2, 0.3) ^f	0.7 (0.6, 0.7)	<.001
Catalase homogenized liver	U/g	15.5 (15, 16) ^b	16 (16, 17) ^{d,e}	6 (6, 7)	12 (11, 12)	<.001
MDA serum	MDA/L	3.15 (3, 3.2) ^b	3 (3, 3.1) ^d	5.15 (5, 5.2)	3.3 (3.2, 3.5)	<.001
MDA homogenized liver	MDA/g	36.2 (0.79) ^{a,b,c}	38.6 (1.26) ^{d,e}	69.2 (1.14) ^f	43.5 (2.17)	<.001
Protein carbonylation serum	nmol/mL	0.63 (0.11) ^{b,c}	0.74 (0.13) ^d	1.53 (0.22) ^f	0.91 (0.15)	<.001
Protein carbonylation	nmol/mg	25.5 (25, 26) ^{b,c}	26 (25, 26) ^{d,e}	55.5 (55, 56)	28 (28, 30)	<.001

Outcome	Units	Study Group				<i>P</i>
Measure		Group 1	Group 2	Group 3	Group 4	Value [†]
carbonylation						
homogenized liver						
Caspase 3	arbitrary					
homogenized liver	unit	5.5 (5.4, 5.6) ^{b,c}	5.6 (5.5, 5.6) ^d	9.45 (9, 9.5)	7.6 (7.5, 7.8)	<.001

[†]Analysis of variance (bolded) or Kruskal-Wallis to test for inter-group differences.

Values are reported as means (SD) or medians (IQR). Group 1 was control, Group 2 was TQ-treated, Group 3 was ATO-treated, and Group 4 was combined ATO/TQ-treated.

The following significant pairwise comparisons were found between groups ($P<.05$): ^aGroup 1 versus Group 2, ^bGroup 1 versus Group 3, ^cGroup 1 versus Group 4, ^dGroup 2 versus Group 3, ^eGroup 2 versus Group 4, and ^fGroup 3 versus Group 4.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATO, atorvastatin; GSH, glutathione; IQR, interquartile range; MDA, malondialdehyde; SD, standard deviation; TQ, thymoquinone.

Figure 1. Violin plots of outcome measures for each study group.

189x105mm (300 x 300 DPI)

Figure 2. Histological examination of hematoxylin and eosin-stained sections of rat liver for all study groups. a. Group 1 (control) showing normal architecture. Note the central vein (C) and hepatic sinusoids (S). b. Group 2 (TQ-treated group) showing normal central vein (C) and hepatic sinusoids (S) similar to that of Group 1. c. Group 3 (ATO-treated group) showing the central vein (C) packed with red blood cells and fluid exudate (arrowheads) and dilated congested sinusoids (S) compressing the surrounding hepatic cell plates. Note the vacuolated cytoplasm (arrows) and the apoptotic cell (P) with disfigured nucleus and increased cytoplasmic inflammatory mononuclear cells (circles). d. Group 3 showing lymph congestion in the central vein (C). Note the dilated sinusoids (S). e. Group 4 (combined ATO/TQ-treated group) showing congested central veins (C) and dilated hepatic sinusoids (arrows); both appear packed with red blood cells. f. Group 4 showing binucleated cells (B) with cytoplasmic vacuoles of variable sizes, one of which was indenting the nucleus (arrowhead). Note the multiple Kupffer cells around the sinusoids (arrows).

250x286mm (300 x 300 DPI)

Figure 3. Histological examination of Masson's trichrome-stained sections of rat liver for all study groups. a.

Group 1 (control) showing minimal connective tissue (arrows) around the central vein (C). There is negligible extension between the surrounding hepatocytes. Note the hepatic sinusoid (S). b. Group 2 (TQ-treated group) showing minimal connective tissue area (arrows) in the hepatic lobule in one field. There is negligible extension between the surrounding hepatocytes. c. Group 3 (ATO-treated group) showing an increase in the amount of connective tissue (arrows) around the central vein (C) and extending between the surrounding hepatocytes (circles). d. Group 4 (combined ATO/TQ-treated group) showing a decrease in the amount of connective tissue (arrow) around the central veins (C) of the hepatic lobules compared to Group 3. There is negligible extension between the surrounding hepatocytes.

241x181mm (300 x 300 DPI)

Figure 4. Ultrastructural examination of rat liver for all study groups. a. Group 1 (control) showing regularly shaped nucleus (N) with finely dispersed chromatin. The rounded or oval mitochondria (M) are defined by 2 limiting membranes and comprised of relatively dense mitochondrial matrix with mitochondrial cristae. The rough endoplasmic reticulum (rER) appears as parallel arrays of fine tubules situated in the cytoplasm on the side of the nucleus. b. Group 2 (TQ-treated group) showing normal euchromatic nucleus (N) with an average number of oval and rounded mitochondria (M) and a few lysosomes (L). c. Group 3 (ATO-treated group) showing numerous enlarged mitochondria (M), discrete fragments of rough endoplasmic reticulum (arrows) with scattered ribosomes (circles), and excess lysosomes (L). Note the degenerated mitochondrion (D) with loss of mitochondrial matrix. d. Group 3 showing numerous enlarged mitochondria (M), fat droplets (F), and a hepatic nucleus (N) with condensed chromatin and irregular nuclear membrane. Note the discrete fragments of rough endoplasmic reticulum (arrows). e. Group 3 showing numerous mitochondria around the nucleus (N), of different shapes and sizes (M), increased number of lysosomes (L), and vacuolated (V), rarified cytoplasm (arrow). f. Group 4 (combined ATO/TQ-treated group) showing restoration in the structure of the cytoplasmic organelles and nuclei; normal nucleus (N), numerous rounded and oval mitochondria (M), and normal rough endoplasmic reticulum (rER) are arranged in parallel tubules to the side of the nucleus.

221x145mm (300 x 300 DPI)