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**Thymoquinone protects rat liver after partial hepatectomy under ischemia/reperfusion through oxidative stress and endoplasmic reticulum stress prevention**

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**Short title:** Thymoquinone effects on partial hepatectomy

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## Abstract

Ischemia reperfusion (I/R) is associated with liver injury and impaired regeneration during partial hepatectomy (PH). The aim of this study was to investigate the effect of thymoquinone (TQ), the active compound of essential oil obtained from *Nigella sativa* seeds, on rat liver after PH.

Male Wistar rats were equally divided into four groups (n=6) receiving an oral administration of either vehicle solution (Sham and PH groups) or TQ at 30 mg/kg (TQ and TQ+PH groups) for ten consecutive days. Then, rats underwent PH (70 %) with 60 min of ischemia followed by 24h of reperfusion (PH and TQ+PH groups). Alanine aminotransferase (ALT) activity and histopathological damage were determined. Also, antioxidant parameters, liver regeneration index, hepatic adenosine triphosphate (ATP) content, endoplasmic reticulum (ER) stress and apoptosis were assessed. In response to PH under I/R, liver damage was significantly alleviated by TQ treatment as evidenced by the decrease in ALT activity ( $P < 0.01$ ) and histological findings ( $P < 0.001$ ). In parallel, TQ preconditioning increased hepatic antioxidant capacities. Moreover, TQ improved mitochondrial function (ATP,  $P < 0.05$ ), attenuated ER stress parameters and repressed the expression of apoptotic effectors. Taken together, our results suggest that TQ preconditioning could be an effective strategy to reduce liver injury after PH under I/R. The protective effects were mediated by the increase of antioxidant capacities and the decrease of ER stress and apoptosis.

## Keywords:

Ischemia reperfusion injury, hepatectomy, thymoquinone, oxidative stress, endoplasmic reticulum stress, apoptosis.

## Introduction

Partial hepatectomy (PH) is considered as the operation of choice for the treatment of many liver malignancies (1, 2) and for living donor liver transplantation (3). However, bleeding remains a major prognostic factor in liver resection. Complete clamping of the hepatic inflow at the hepatoduodenal ligament (the Pringle maneuver) is a safe strategy to alleviate this problem (4). This maneuver, although effective in preventing excessive blood loss, is complicated by ischemia/reperfusion (I/R) injury, which compromises functional recovery of the remnant liver (4, 5). Clinically, liver I/R injury results in elevated liver transaminases, bile duct stricture and even liver failure (4-6). Furthermore, other organs can develop dysfunction secondary to the liver damage such as lungs, heart and kidneys (7). Besides I/R, the surgical resection itself activates various complex cell signaling cascades of which many sides are still unresolved (8, 9). For example, reactive oxygen species (ROS) are involved in the modulation of several signaling pathways that may influence liver regeneration after PH (10, 11). Also, mitochondria have been shown to be an important player of liver regeneration (12). Actually, high energy demand on the hepatocytes is required during the regenerative process after PH and this energy is provided by mitochondria through oxidative phosphorylation. In return, mitochondria release a large amount of ROS (13, 14) which activate proteins that inhibit the cell cycle (15, 16). In addition, oxidative stress causes damage to cell components including proteins, lipids, and DNA resulting in cellular disorders and further organelle malfunction (17).

Endoplasmic reticulum (ER) stress is triggered in several pathological events such as hypoxia, glucose deprivation and oxidative stress. ER homeostasis disruption leads to the accumulation of unfolded and misfolded proteins in the ER lumen. As a consequence, unfolded protein response (UPR) is activated in order to resolve this protein-folding defect and so to restore ER homeostasis (18). However, if the UPR is insufficient to alleviate the stress, caspase

dependent and independent cell death occurs (19). Indeed, ER stress activates caspase 12 and mitochondrial apoptosis pathway (20, 21). ER stress also upregulated glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) and voltage-dependent anion channel (VDAC) proteins which initiate the efflux of cytochrome C from the outer mitochondrial membrane, which in turn, activates the pro-apoptotic proteins caspase 9 and its effectors caspase 3 (22).

Thymoquinone (TQ) (2-isopropyl-5-methylbenzo-1, 4-quinone), is the major and the main active constituent of the essential oil of *Nigella sativa* seeds (23). TQ has several biological activities, especially antioxidant and free radical scavenging capacity (24, 25). Recently, it has been shown that TQ protects rat liver against I/R injury (20). However, its effect on liver subjected to resection under I/R is still unknown. Therefore, this study aimed to evaluate the effect of TQ administration in rat before a 2/3 hepatectomy (PH).

## Results

In order to explore liver injury, we evaluated whether TQ pretreatment could preserve liver architecture and hepatocyte proliferation capacity after PH (Figure 1). As expected, a disorganized hepatic lobule aspect with focal necrosis was observed in rat livers subjected to PH under I/R. TQ treatment markedly attenuated the hepatic damage when compared to PH group ( $2.3 \pm 0.3$  vs.  $3.9 \pm 0.4$ ,  $P < 0.001$ ). We noted a relatively preserved hepatic architecture with less vacuolization and nuclear picnosis, and few zones of necrosis were detected. Consistent with these observations, we found that ALT activity in serum of PH+TQ group was significantly decreased when compared to PH group. Values reached  $161 \pm 42$  and  $74 \pm 46$  U/L for PH and TQ+PH groups, respectively ( $P < 0.01$ ). We noted also that TQ treatment in sham operated rats (TQ group) did not result in any modification in ALT activity in comparison to sham group. In addition, hepatocyte proliferation, assessed 24 hours after PH by quantitation of the number of Ki-67-positive hepatocytes, was greater in TQ+PH group

than in PH group ( $7.7 \pm 5.8$  vs.  $16.0 \pm 3.6$  %,  $P < 0.01$ ). Thus, we could assume that the administration of TQ decreased hepatic damage and promoted liver regeneration.

It is well known that PH is associated with ROS production. We therefore examined the possible involvement of TQ in stimulating antioxidant systems. As shown in figure 2, PH under I/R resulted in a significant reduction in the antioxidant enzyme activities and increase in oxidative stress parameters. Interestingly, when compared to PH group, rats pre-treated with TQ had significantly increased activity of glutathione peroxidase (GPX) ( $271 \pm 43$  vs.  $209 \pm 21$   $\mu\text{mol oxidized GSH}/\text{min}/\text{mg prot}$ ,  $P < 0.05$ ), superoxide dismutase (SOD) ( $5.8 \pm 0.2$  vs.  $5.0 \pm 0.4$   $\text{U}/\mu\text{g prot}$ ,  $P < 0.05$ ) and catalase (CAT) ( $140 \pm 18$  vs.  $96 \pm 18$   $\mu\text{mol H}_2\text{O}_2/\text{min}/\text{mg prot}$ ,  $P < 0.01$ ), sulfhydryl proteins (SHP) ( $10 \pm 1$  vs.  $6 \pm 3$   $\mu\text{g}/\text{mg prot}$ ,  $P < 0.05$ ) whereas they had reduced malondialdehyde (MDA) ( $0.37 \pm 0.08$  vs.  $0.70 \pm 0.15$   $\text{nmol}/\text{mg prot}$ ,  $P < 0.05$ ) and conjugated dienes (CD) ( $0.04 \pm 0.02$  vs.  $0.07 \pm 0.02$   $\text{nmol}/\text{mg prot}$ ,  $P < 0.05$ ) concentrations. TQ treatment without PH did not result in any modification of oxidative stress parameters.

Given the central role for mitochondria to supply energy in cell, we assessed ATP concentration in livers (Figure 3). Our data showed that livers following ischemia and PH had a significant decrease in ATP content compared to livers from sham rats ( $0.09 \pm 0.02$  vs.  $0.71 \pm 0.10$   $\mu\text{mol}/\text{mg prot}$ ,  $P < 0.001$ ). However, pre-treatment with TQ enabled to preserve ATP content in comparison to PH group ( $0.19 \pm 0.07$   $\mu\text{mol}/\text{mg prot}$ ,  $P < 0.05$ ).

In order to evaluate whether the hepatoprotective effect of TQ could be related to ER stress prevention, protein relative expression of activating transcription factor-4 (ATF4), activating transcription factor-6 (ATF6) and X-box-binding protein-1 (XBP1) and protein concentration of glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) were assessed (Figure 4). As expected, ischemia and PH markedly intensified the activation of all

ER stress proteins when compared to sham group. As regarded to PH group, TQ pretreatment induced a significant drop in the expression of ATF4 ( $P < 0.01$ ), ATF6 ( $P < 0.01$ ) and XBP1 ( $P < 0.01$ ) proteins and in the concentration of GRP 78 ( $519 \pm 229$  vs.  $1143 \pm 350$   $\mu\text{g/mL}$ ,  $P < 0.001$ ) and CHOP ( $280 \pm 113$  vs.  $490 \pm 60$   $\mu\text{g/mL}$ ,  $P < 0.001$ ).

Given that ER stress and mitochondrial dysfunction promote cell apoptosis (20, 26, 27), we explored apoptosis proteins after PH under I/R (Figure 5). In concordance with the previous results, ischemia and PH increased apoptosis when compared to sham operated rats. Importantly, the apoptosis effectors were down-regulated by pretreatment with TQ. We found  $289 \pm 80$  vs.  $832 \pm 327$   $\text{ng/mL}$  ( $P < 0.001$ ),  $4.23 \pm 1.29$  vs.  $9.57 \pm 1.58$  ( $P < 0.001$ ),  $4.76 \pm 0.84$  vs.  $9.54 \pm 1.98$  ( $P < 0.001$ ) and  $7.36 \pm 1.12$  vs.  $16.37 \pm 2.35$   $\text{pg/mg prot}$  ( $P < 0.001$ ) for cytochrome c, caspase-9, caspase-12 and caspase-3 activities, respectively.

## Discussion

This study demonstrates for the first time that TQ protects rat liver from injury caused by hepatectomy under I/R and promoted hepatic regeneration through the induction of antioxidant defense capacity, the prevention of oxidative stress and the attenuation of ER stress, mitochondrial damage and apoptosis.

It was previously reported that TQ has many pharmacological actions, including antioxidant and anti-inflammatory effects against several hepatotoxic molecules (28-31). Moreover, it has been shown that TQ suppresses liver fibrosis (32, 33) and attenuates liver injury induced by ischemia (20, 34). Nevertheless, TQ effects have not been examined in an experimental model combining PH and I/R.

Partial hepatectomy under IR increased liver injury as evidenced by ALT release in serum. Besides, histopathological evaluation of the livers has confirmed liver damage. In contrast, rats pretreated with TQ before PH showed a reduction in liver injury and an improvement of

the histopathological abnormalities. These findings consolidate previous reported works showing that TQ protects rat liver against IR injury (20, 34). In line with the decrease of liver injury, we found that TQ promoted liver regeneration. This is consistent with previous reports showing that TQ had healing effects in bone (35) and wound.

Thus far, the hepatoprotective effect of TQ is largely ascribed to its antioxidative properties. Imbalance between oxidants and antioxidants is commonly termed as oxidative stress. In response to this state, cells react by their antioxidant defense machinery including both non-enzymatic and enzymatic antioxidants (36). In our study, we found that PH under IR led to a decreased of SOD, GPx and CAT activities, of SHP concentration while it was accompanied by an increased MDA and CD contents. These results are in line with those of previous studies suggesting that free radical damage occurs in the early phase of liver regeneration (37) resulting in lipid peroxidation (38) and mitochondrial glutathione depression (39). Furthermore, we noticed that TQ attenuated PH injury by preserving the anti-oxidant enzyme activities and enhancing the level of SHP. As a result, lipid peroxidation evidenced by both MDA and CD concentrations were lessened. In fact, the antioxidant effect of TQ has been assigned to its ability to scavenge free radical (40, 41) and to up-regulate antioxidant gene expression (42, 43). Moreover, the action of TQ could be promoted by its unrestricted crossing of membrane barriers to access subcellular compartments and thus to protect them (25).

Further alterations touching cell organelles, including mitochondria, could be generated as a result of excessive ROS production. Previous data have hypothesized that oxidative stress induced by PH under IR impairs mitochondrial function and alters cell energy metabolism (37, 39). Moreover, hepatic regeneration could be affected by the change in mitochondrial energy production (44, 45). As previously reported, our study confirms the fact that PH under I/R decreases ATP production. In contrast, pre-treatment of rats with TQ led to the



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preservation of ATP stores in liver, which is in concordance with other works (46, 47). Thus, we can presume that the prevention of ATP drop would be due, at least in part, to the ability of TQ to preserve mitochondrial integrity.

The formation of ROS and the induction of ER stress are known to be closely linked processes (48). As a consequence of ER dysfunction, a signal transduction cascade which progress from the ER lumen to the cytoplasm and nucleus known as the UPR is activated (18). The UPR is an adaptive attempt that aims at restoring the ER homeostasis. It is mediated by three signaling proteins located at the ER membrane termed protein kinase RNA-like ER kinase (PERK), inositol requiring 1 (IRE1) and ATF6. The activation of these UPR sensors is impeded by their binding with a luminal protein chaperone, the glucose regulated protein 78 (GRP78). Once released from GRP78, the luminal domain of IRE1 dimerizes and trans-autophosphorylates and is thus transformed into an activated form. Activated IRE1 produces a potent transcription factor XBP1 by cleavage of XBP1 mRNA. The activated XBP1 up-regulates UPR genes by directly binding to their related promoters (49). During ER stress, PERK is activated by a similar mechanism to IRE1. Activated PERK phosphorylates eukaryotic initiation factor 2 (eIF2 $\alpha$ ). Phosphorylated eIF2 $\alpha$  attenuates global protein synthesis by preventing ribosomal initiation complexes formation. Meanwhile, eIF2 $\alpha$  is necessary for translation of some mRNAs, including the mRNA that encodes the ATF4. ATF4 regulates several UPR target genes principally those engaged in antioxidative stress responses, autophagy and ER stress-induced apoptosis such as CHOP (50, 51). Upon GRP78 release, ATF6 translocates from the ER to the Golgi where it is cleaved to form an active transcription factor. Active ATF6 migrates then to the nucleus and up-regulates numerous ER chaperone genes including GRP78, GRP94, and CHOP (52). Our data revealed the activation of all three branches of UPR (PERK, ATF6, IRE1) and their downstream targets ATF4, ATF6 $\alpha$ , XBP1, respectively. We also showed the presence of ER stress in PH under IR

condition by the induction of CHOP and the up-regulation of GRP78. These results are in line with those of a previous report which highlighted ER stress after PH (21). In our study, we demonstrated that the induction of ER stress parameters in response to PH under IR condition could be reduced by TQ pre-treatment. Our data are in keeping with our previous findings showing that TQ reduces ER stress induced by warm liver IR in rat (20). However, how TQ protects ER from PH under I/R damage remains unclear; we presume that it could be related to the attenuation of oxidative stress.

Mitochondrial damage and ER stress induction by PH under I/R condition prompted us to further evaluate apoptosis. In the present study, an increase of cytochrome C release was observed after PH. In addition, both caspase-9 and -3 were found activated which indicates that PH under I/R may stimulate apoptotic cell death (53, 54). In parallel, our data showed a caspase-12 induction, which is known as an important protein implicated in ER-induced apoptosis (55). In our experimental model, TQ induced down-regulation of cytochrome C release and a marked decrease in the expression of caspase-9, -12 and -3. These findings are in line with a previous research paper (34). The anti-apoptotic effect of TQ could be attributed to its potent scavenger and antioxidant role that help to attenuate ER stress and further apoptosis.

In summary, this study is the first to evaluate the hepatoprotective effects of TQ in an experimental model of PH. Data reported here suggest that TQ plays an important role in the prevention of liver injury in conditions of hepatectomy under I/R. TQ protection is related to its capacity to prevent oxidative stress, mitochondrial damage, ER stress and apoptosis.

## **Materials and methods**

### ***Experimental animals and Ethics Statement***

Male Wistar rats weighing 200-230 g were used in this study. They were housed in an air-controlled room with 12 h light and dark cycles, and a constant temperature ( $22 \pm 2^\circ\text{C}$ ). They had free access to food and water. All procedures were carried out in accordance with the European Union Regulations (Directive 2010/63/EU) for animal experiments and approved by the local Experimental Animals Ethics Committee of the Faculty of Pharmacy of Monastir.

### ***Surgical Procedure***

Rats were anaesthetized with an intra-peritoneal (ip) injection of pentobarbital (5 %), and then subjected to PH (70 % of liver parenchyma) under 60 min of ischemia as described previously (21). Briefly, after dividing the ligaments of the hepatic lobes and resection of their left hepatic lobe, rats underwent 60 min of partial liver warm ischemia by clamping their portal triad supplying the median lobe. At the end of ischemia, the right and caudate lobes were resected, and reperfusion of the median lobe was achieved by the release of the clamp. Sham operated rats was subjected to anesthesia and pedicle dissection without resection or ischemia. After surgery, rats were allowed food and water *ad libitum*. Blood and liver samples were collected after 24 h reperfusion and samples were stored at  $-80^\circ\text{C}$ .

### ***Experimental groups***

Rats were allocated at random into four experimental groups, with six rats in each group:

Group 1 (Sham group): Rats were subjected to only laparotomy without occlusion of hepatic pedicle and PH.

Group 2 (TQ group): rats were orally pretreated with TQ (30 mg/kg) for ten consecutive days (20). Rat livers were then subjected to laparotomy without occlusion of hepatic pedicle and PH.

Group 3 (PH group): rats underwent 70% hepatectomy under 60 min of ischemia followed by 24 h of reperfusion.

Group 4 (TQ+PH group): rats were orally pretreated with TQ (30 mg/kg) for ten consecutive days (20). Rat livers were then subjected to surgery as in group 3.

### ***Chemicals***

TQ (Sigma Aldrich) was dissolved in ethanol and aliquoted and stored at -20°C. Working solution was prepared daily before oral administration by diluting stock solution with distilled water (1:4). Ten days prior the surgery, sham and PH animals underwent a daily intragastric administration of vehicle solution (ethanol and distilled water) as described previously (20).

### ***Histology and Immunohistochemistry***

Liver biopsies were fixed in 10 % formalin solution, embedded in paraffin and cut at 4 µm thickness. To appraise the severity of hepatic injury, stained sections with hematoxylin and eosin were graded with a point-counting method on a scale from 1 (excellent) to 5 (poor) as described previously (56): (1) normal rectangular structure, (2) rounded hepatocytes with an increase of the sinusoidal spaces, (3) vacuolization, (4) nuclear picnosis and (5) necrosis. Damage score was estimated semi-quantitatively by an experienced pathologist without having knowledge about the treatment groups.

For liver regeneration, liver samples were immunostained with a rabbit monoclonal antibody against Ki 67 (clone SP6, Abcam, Cambridge, MA), developed with diaminobenzidine, and counterstained with hematoxylin (57).

### ***Transaminase determination***

The serum activity of alanine aminotransferase (ALT) was determined using a commercial kit from DiaSys (Diagnostic System, Germany) following supplier's instructions.

### ***Determination of hepatic adenosine triphosphate content***

Hepatic adenosine triphosphate (ATP) measurements were performed using a firefly bioluminescence assay kit (ATP Bioluminescent Assay Kit FLAA-1KT, Sigma Aldrich St Quentin Fallavier, France) as described elsewhere (58).

#### ***Determination of oxidative stress parameters***

Liver tissues were homogenized in ice-cold phosphate buffered saline (100 mM KH<sub>2</sub>PO<sub>4</sub>, 100 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.4) to estimate the content of SHP and the activities of GPx, SOD and CAT. For MDA determination, tissues homogenization was carried out in ice-cold tris-buffered saline (100 mM Tris, pH 7). For CD measurement, liver tissues were homogenized in distilled ice-water.

Liver GPx activity was estimated by the method of Floche and Gunzler 1984 (59). SOD activity assay was performed using the method previously described by Marklund and Marklund (60). CAT activity was determined according to Clairbone method (61). The determination of SHP level was achieved in agreement to the method of Sedlak (62). MDA was measured by the thiobarbituric acid (TBA) assay (63). CD was assessed according to Srinivasan (64).

#### ***Determination of endoplasmic reticulum stress parameters***

We looked for ER stress through the determination of GRP78 and CHOP using ELISA kits (MyBioSource, San Diego, CA) according to the manufacturer's protocol and by the determination of protein relative expression of ATF6 $\alpha$ , ATF4 and XBP1 by western blot technique.

Briefly, liver tissues were homogenized in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM NaF, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 0.1 mM orthovanadate, 0.05% Triton-X 100 and 2% protease inhibitor cocktails). Equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes as described elsewhere (65). Then, membranes were incubated over night at 4°C with primary antibodies

for ATF6 $\alpha$  (sc-22799), ATF4 (sc-101663), XBP1 (sc-7160) and Hsc70 (sc-7298) acquired from Santa Cruz Biotechnology. After washing, the membranes were incubated for 2h at room temperature with appropriate horseradish peroxidase conjugated secondary antibody. Signals were detected by enhanced chemiluminescence and quantified by the quantity one software program (Bio-Rad Laboratories, Hercules, CA, USA).

#### ***Determination of apoptosis parameters***

Cytochrome C level was evaluated using ELISA kit from MyBioSource (San Diego, CA). A fluorometric assay kit was used (Biovision Palo Alto, CA) to determine liver caspase 12 level. Caspase 9 and caspase 3 levels were measured using a colorimetric assay kit from Biovision (Palo Alto, CA).

#### ***Statistical analysis***

Data were expressed as mean  $\pm$  standard deviation (SD) and were compared statistically using Graph Pad Prism software (version 6.01) by variance of analysis (ANOVA) followed by Newman-Keuls multiple comparison test. P-value of less than 0.05 was considered statistically significant.

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The authors declare that they have no conflicts of interests concerning this article.

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## Figure legends:

**Figure 1.** Thymoquinone prevented liver injury and promoted regeneration after PH.

(a) H&E staining of the liver section in the Sham, PH and TQ+PH groups. Arrows pointed out the necrotic areas. Scale bar represents 100 $\mu$ m, (b) damage score, (c) ALT activity in plasma, (d) immunostaining of Ki67 of the liver section in the PH and TQ+PH groups. Scale bar represents 300 $\mu$ m and (e) representative immunohistochemical analysis showing the percentage of Ki67 positive cells. Results are expressed as mean  $\pm$  SD (n = 6 for each group). \*P<0.05 versus Sham. +P<0.05 versus TQ. #P<0.05 versus PH.

**Figure 2.** Thymoquinone pretreatment attenuated oxidative stress after PH under I/R.

Liver samples were assessed for MDA concentration (a), glutathione peroxidase activity (b), superoxide dismutase activity (c), catalase activity (d), sulfhydryl proteins content (e) and conjugated dienes concentration (f) in livers harvested from Sham, TQ, PH and TQ+PH groups. Results are expressed as mean  $\pm$  SD (n = 6 for each group). \*P<0.05 versus Sham. +P<0.05 versus TQ. #P<0.05 versus PH.

**Figure 3.** Thymoquinone increased ATP content in liver after PH.

ATP concentration in liver tissue of Sham, PH and TQ+PH groups. Results are expressed as mean  $\pm$  SD (n = 6 for each group). \*P<0.05 versus Sham. #P<0.05 versus PH.

**Figure 4.** Thymoquinone preconditioning attenuated ER stress.

GRP78 (a) and CHOP (b) concentration determined by ELISA kits and the relative expression of ATF6 (c), ATF4 (d) and XBP1 (e) proteins level evaluated by western blot in Sham, PH and TQ+PH groups. Results are expressed as mean  $\pm$  SD (n = 6 for each group). \*P<0.05 versus Sham. #P<0.05 versus PH.

**Figure 5.** Thymoquinone pretreatment decreased liver apoptosis after PH.

Caspase 12 (a), Cytochrome C (b) Caspase 9 (c) and Caspase 3 (d) concentrations were determined. Livers were harvested from Sham, PH and TQ+PH groups. Results are expressed as mean  $\pm$  SD (n = 6 for each group). \*P<0.05 versus Sham. #P<0.05 versus PH.

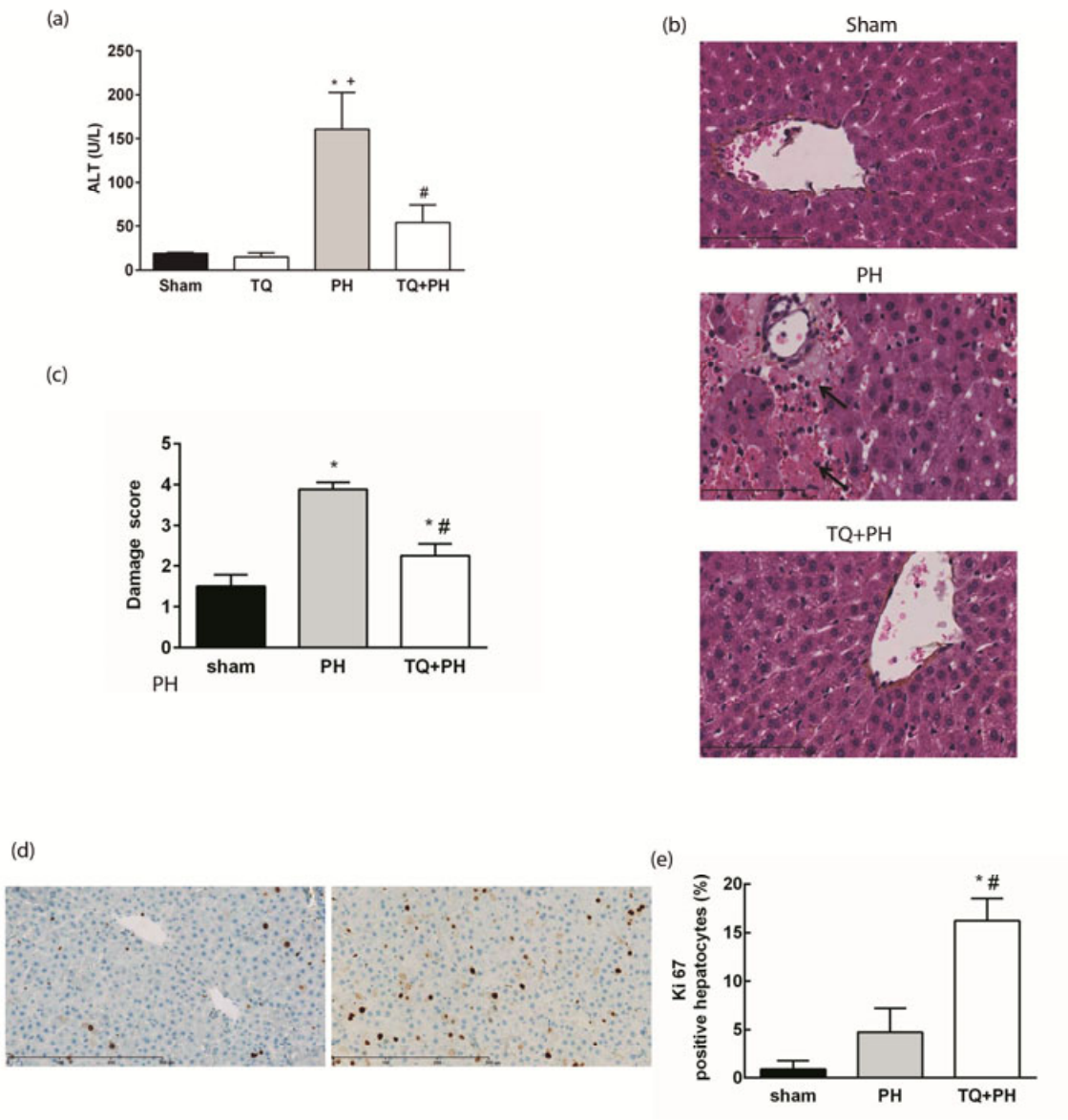


Figure 1

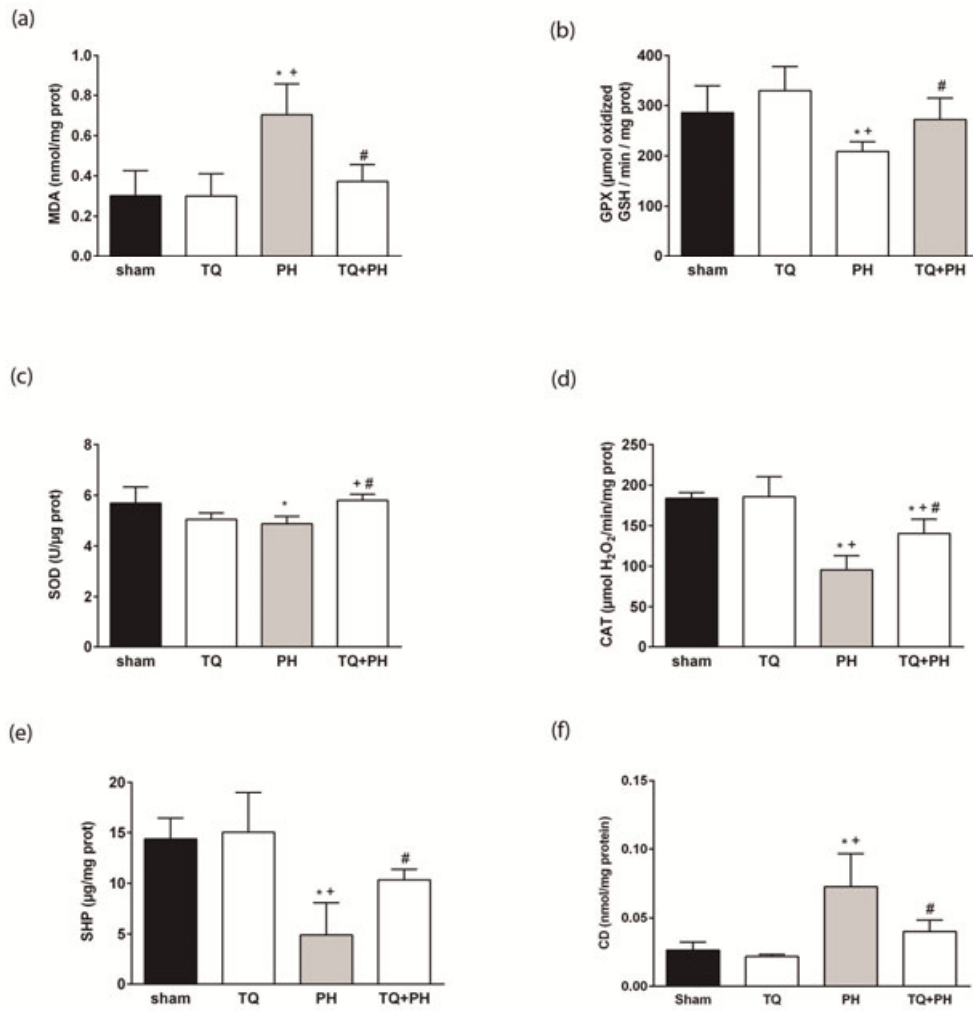


Figure 2

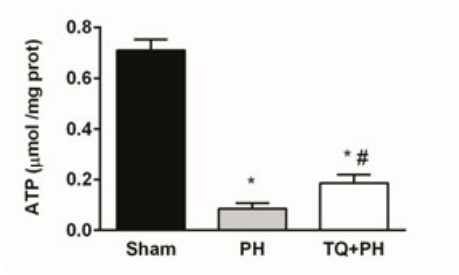


Figure 3

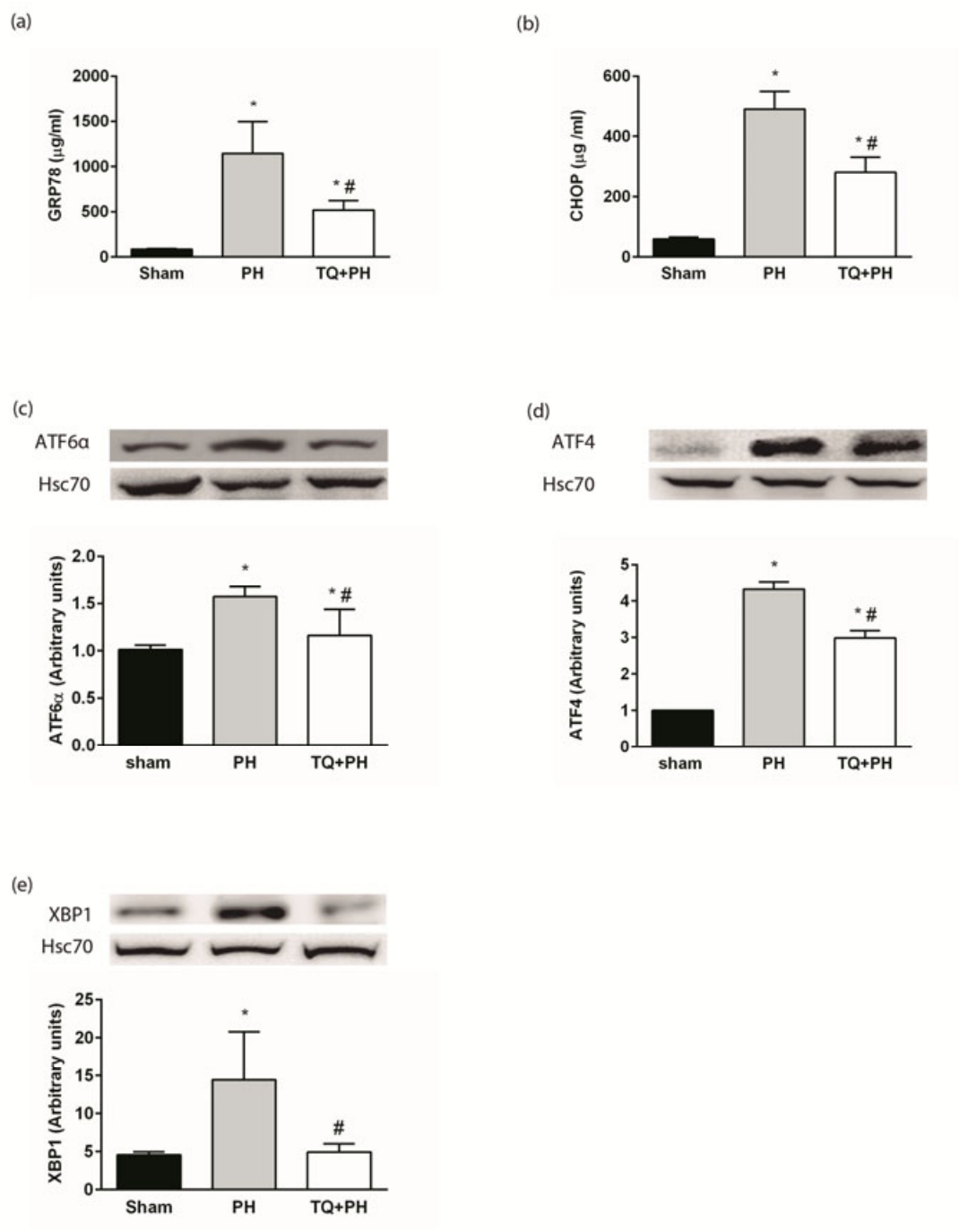
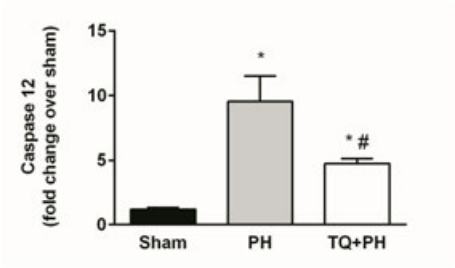
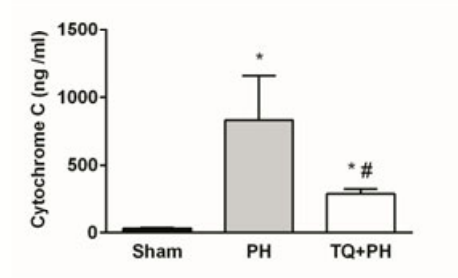


Figure 4

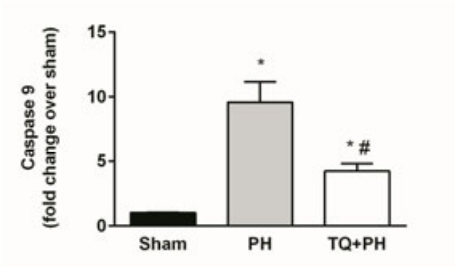
(a)



(b)



(c)



(d)

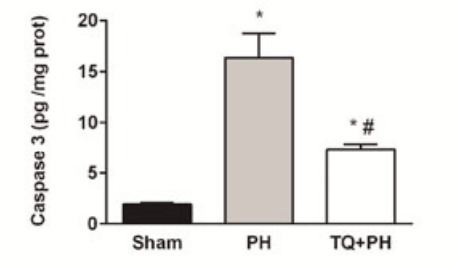


Figure 5