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RESEARCH ARTICLE



Exacerbated liver injury of antithyroid drugs in endotoxin-treated mice

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ABSTRACT

Drug-induced liver injury is a major concern in clinical studies as well as in post-marketing surveillance. Previous evidence suggested that drug exposure during periods of inflammation could increase an individual's susceptibility to drug hepatotoxicity. The antithyroid drugs, methimazole (MMI) and propylthiouracil (PTU) can cause adverse reactions in patients, with liver as a usual target. We tested the hypothesis that MMI and PTU could be rendered hepatotoxic in animals undergoing a modest inflammation. Mice were treated with a nonhepatotoxic dose of LPS (100 µg/kg, i.p) or its vehicle. Nonhepatotoxic doses of MMI (10, 25 and 50 mg/kg, oral) and PTU (10, 25 and 50 mg/kg, oral) were administered two hours after LPS treatment. It was found that liver injury was evident only in animals received both drug and LPS, as estimated by increases in serum alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and TNF-α. An increase in liver myeloperoxidase (MPO) enzyme activity and tissue lipid peroxidation (LPO) in addition of liver glutathione (GSH) depletion were also detected in LPS and antithyroid drugs cotreated animals. Furthermore, histopathological changes including, endotheliitis, fatty changes, severe inflammatory cells infiltration (hepatitis) and sinusoidal congestion were detected in liver tissue. Methyl palmitate (2 g/kg, i.v, 44 hours before LPS), as a macrophage suppressor, significantly alleviated antithyroids hepatotoxicity in LPS-treated animals. The results indicate a synergistic liver injury from antithyroid drugs and bacterial lipopolysaccharide coexposure.

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Introduction

Drug-induced liver injury (DILI) is an important challenge associated with many pharmaceuticals (Abboud and Kaplowitz 2007). Methimazole (MMI) and propylthiouracil (PTU) are antithyroid drugs which their administration is associated with liver injury in humans (Cooper 1999, 2005). The MMI-induced liver injury is a rare but serious clinical complication (Bandyopadhyay *et al.* 2002, Yang *et al.* 2015a, 2015b). Several cases of liver injury have been reported after MMI administration (Woeber 2002, Gallelli *et al.* 2009, Jeon *et al.* 2012). PTU is also associated with severe and fatal hepatotoxicity especially in pediatrics (Rivkees and Mattison 2009, Carrion *et al.* 2010). The precise mechanism(s) of antithyroid drugs-induced hepatotoxicity is unknown so far (Heidari *et al.* 2012a, 2015b).

Recently, an intriguing animal model for evaluating drug-induced hepatic injury has been developed (Roth and Ganey 2011, Webb and Adams 2015). It has been found that a mild and nontoxic inflammation could drastically enhance the hepatotoxicity induced by a wide range of drugs (Luyendyk *et al.* 2003, Ganey *et al.* 2004, Su *et al.* 2014, Niknahad *et al.* 2016).

Although the precise mechanism(s) of inflammation/drug interaction to induce hepatic injury is less clear, several studies proposed a role for inflammatory cytokines, oxidative stress, and drugs reactive intermediates in this situation (Antoine *et al.* 2008, Deng *et al.* 2009).

Cytochrome P450 enzyme (CYP450) and flavoprotein-mixed-function oxidase (FMO) are suspected to be involved in the hepatic metabolism of MMI (Poulsen *et al.* 1974, Lee and Neal 1978). Glyoxal and N-methyl thiourea are two suspected reactive metabolites of MMI which might be involved in the liver injury induced by this drug (Mizutani *et al.* 2000). The metabolic pathway for PTU is less known in the liver. To date, there is no report on the PTU reactive metabolite(s) formation in the liver, and the role of such intermediates in the hepatotoxicity induced by this drug is ambiguous. Some investigations proposed that reactive metabolites are produced during myeloperoxidase (MPO) action on PTU in neutrophils, which might be related to agranulocytosis as a deleterious side effect of this drug (Lee *et al.* 1988, Waldhauser and Uetrecht 1991). However, the production of such metabolites in the liver has not been proven yet. Hence, more future experiments are needed to elucidate the

mechanism(s) of PTU-induced liver injury and the contribution of drug reactive metabolite(s) in this complication.

The current study aimed to investigate the hepatotoxic profile of antithyroid drugs in animals with a mild and noninjurious inflammation. For this purpose, mice were pretreated with lipopolysaccharide (LPS), and then animals were challenged with non-hepatotoxic doses of antithyroid drugs. Plasma concentration of TNF- α , ALT, LDH, AST and bilirubin in addition of liver tissue histopathological changes, lipid peroxidation (LPO), hepatic glutathione (GSH) content, and myeloperoxidase (MPO) activity were monitored to evaluate if LPS hasten antithyroid drugs hepatotoxicity.

Material and methods

Chemicals

Methimazole was purchased from Medisca Pharmaceutique (Montreal, Canada). n-Butanol and 5,5'-dithio-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich (St. Louis, USA). Thiobarbituric acid (TBA) was obtained from Servia (Heidelberg, New York). Ortho-phosphoric acid (OPA), trichloroacetic acid (TCA), propylthiouracil (PTU), methyl palmitate, ethylenediaminetetraacetic acid (EDTA), methanol and hydroxymethyl aminomethane hydrochloride (Tris-HCl) were purchased from Merck (Darmstadt, Germany). Bacterial lipopolysaccharide (LPS) from *E. coli* was purchased from Iranian Biological Resource Center (IBRC) (Tehran, Iran). Mouse TNF- α kit was obtained from eBioscience. Kits for analyzing plasma level of liver injury biomarkers were obtained from Pars Azmun[®] (Tehran, Iran). All salts for preparing buffer solutions were of the highest grade commercially available and prepared from Merck (Darmstadt, Germany).

Animals

Male BALB/c mice (20–35 g weight) ($n=85$), were obtained from Animals Breeding Center, Shiraz University of Medical Sciences (Shiraz, Iran). Mice were housed in cages on woodchip bedding at an ambient temperature of $23 \pm 2^\circ\text{C}$, relative humidity of $\approx 40\%$. Animals had free access to food and water. Mice received humane care and were handled according to the animal handling protocol of Shiraz University of Medical Sciences, approved by a local ethics committee (#92-01-36-6829).

Experimental setup

Mice were randomly allotted into 17 groups ($n=5$ for each group). The treatments were as follow: (A) Control (vehicle-treated); (B) LPS (100 $\mu\text{g/kg}$, i.p); (C) MMI (10 mg/kg, gavage); (D) MMI (25 mg/kg, gavage); (E) MMI (50 mg/kg, gavage); (F) PTU (10 mg/kg, gavage); (G) PTU (25 mg/kg, gavage); (H) PTU (50 mg/kg, gavage); (I) LPS (100 $\mu\text{g/kg}$, i.p) + MMI (10 mg/kg, gavage); (J) LPS (100 $\mu\text{g/kg}$, i.p) + MMI (25 mg/kg, gavage); (K) LPS (100 $\mu\text{g/kg}$, i.p) + MMI (50 mg/kg, gavage); (L) Methyl palmitate (2 g/kg, i.v); (M) Methyl palmitate (2 g/kg, i.v) + LPS (100 $\mu\text{g/kg}$) + MMI (50 mg/kg, gavage); (N) LPS (100 $\mu\text{g/kg}$) + PTU (10 mg/kg, gavage); (O) LPS (100 $\mu\text{g/kg}$) + PTU (25 mg/kg, gavage); (P) LPS (100 $\mu\text{g/kg}$) + PTU (50 mg/kg,

gavage); (Q) Methyl palmitate (2 g/kg, i.v) + LPS (100 $\mu\text{g/kg}$, i.p) + PTU (50 mg/kg, gavage).

The dose of MMI and PTU was selected based on previous investigations in this field (Heidari *et al.* 2014a, 2014b, Karamikhah *et al.* 2015).

For the induction of liver inflammation, bacterial LPS (100 $\mu\text{g/kg}$, i.p) (Mignon *et al.* 1999, Luyendyk *et al.* 2004) was administered 2 hours before drug administration. Kupffer cells inhibition was achieved by administration of methyl palmitate (2 g/kg, i.v; 44 hours before LPS treatment) (Rizzardini *et al.* 1998). Liver injury biomarkers were assessed 24 hours after MMI and/or PTU administration in all experimental groups.

Plasma biochemical analysis and liver histopathology

Animals were anesthetized with pentobarbital (50 mg/kg, i.p) and blood samples were collected from the abdominal *vena cava* under. Animals' liver was removed and washed with sodium chloride (0.9% (w/v; 4°C). Blood was carefully transformed to anticoagulant-coated tubes (VACUETTE[®] Blood Collection Tubes; Greiner Bio-One; Italy), and plasma was prepared by centrifugation (3000 g , 20 min, and 4°C). Plasma was stored at -80°C for further analysis of biomarkers of liver injury including total bilirubin, ALT, LDH, AST as well as plasma TNF- α level. For histopathological assessments, samples of liver were fixed in a buffered formalin solution (0.64% sodium phosphate dibasic, Na_2HPO_4 , 0.4% sodium phosphate monobasic, NaH_2PO_4 , and 10% formaldehyde in double distilled water). Paraffin-embedded sections (5 μm) of liver tissue were prepared and stained with hematoxylin and eosin (H&E) before light microscope viewing (Moezi *et al.* 2013).

Liver glutathione (GSH) content

The hepatic GSH content was assessed according a previously reported procedure using the Ellman reagent (DTNB) (Sedlak and Lindsay 1968). Briefly, samples of liver tissue (200 mg) were homogenized in 8 ml of EDTA solution (20 mM; 4°C). Then, 5 mL of liver homogenate was mixed with 1 mL of trichloroacetic acid (50%, w/v) and 4 mL of double distilled water (4°C). The mixture was vortexed and then centrifuged (765 g , 15 minutes, 4°C). Afterward, 2 mL of supernatant was added to 4 mL of Tris-HCl buffer (40 mM; pH=8.9) and 100 μl of DTNB solution (10 mM in methanol) (Sedlak and Lindsay 1968, Heidari *et al.* 2015a). Finally, the absorbance of developed color was measured at $\lambda=412\text{ nm}$ using an Ultrospec 2000[®]UV spectrophotometer (Uppsala Sweden).

Myeloperoxidase (MPO) activity in liver tissue

The MPO activity in liver tissue was assessed as an index of tissue inflammatory cells infiltration and accumulation. Liver samples (500 mg) were homogenized in 5 mL of hexadecyl-trimethyl-ammonium bromide (HTAB) solution (0.5% w/v; dissolved in 50 mM potassium phosphate buffer; pH = 6) and then centrifuged (3000 g for 20 min at 4°C). Afterward, 100 μl of the supernatant was added to 2.9 mL of potassium phosphate buffer (50 mM; pH=6; containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% of hydrogen

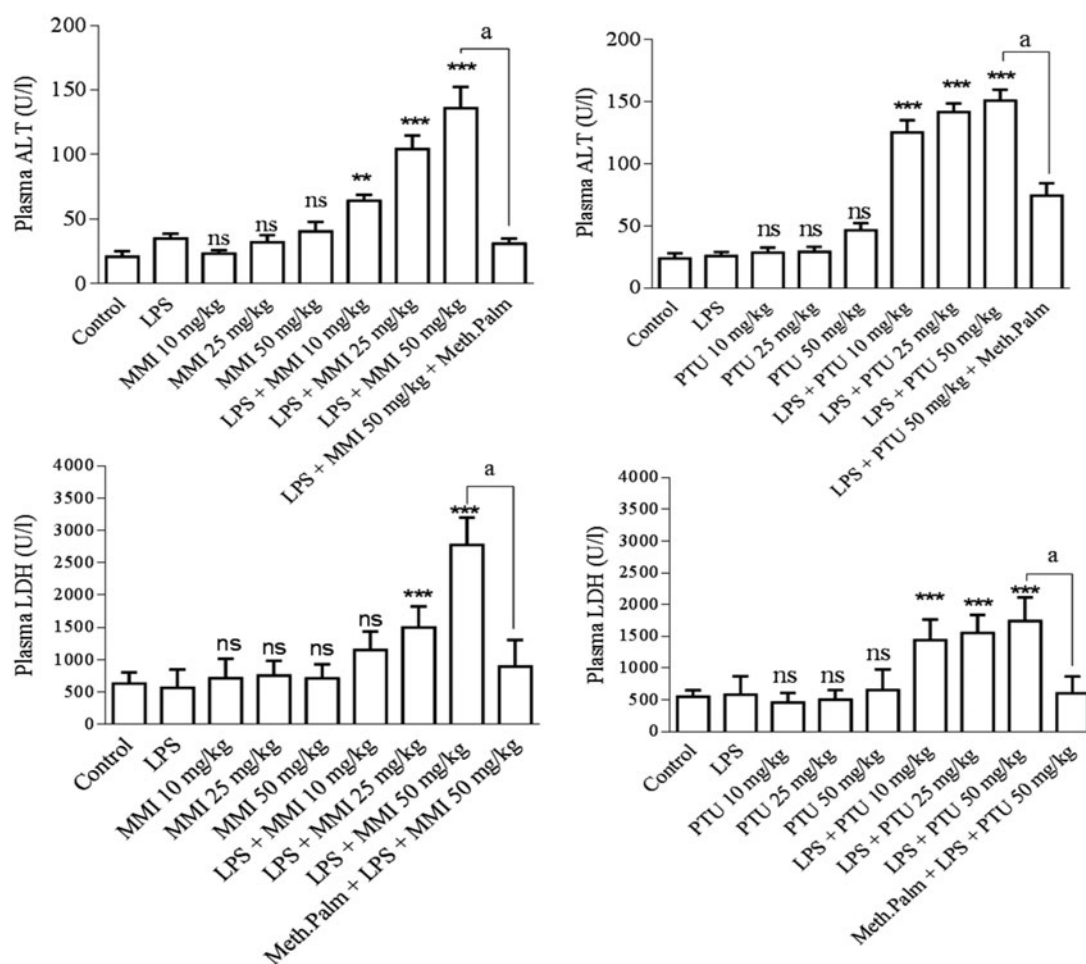


Figure 1. Mice plasma ALT and LDH levels in different experimental groups. Data are shown as Mean $\pm$ SEM ($n = 5$). MMI: MMI; PTU: propylthiouracil; LPS: lipopolysaccharide; Meth.Palm: Methyl palmitate. LPS (100 μ g/kg, i.p) was administered 2 hours before MMI and/or PTU. Methyl palmitate (2 g/kg, i.v) was administered 72 hours before LPS treatment. ns: not significant as compared to control group. **Indicates significantly different values as compared to control animals ($p < 0.01$). ***Indicates significantly different values as compared to control animals ($p < 0.001$). ^aIndicates significantly different values as compared to methyl palmitate-treated groups ($p < 0.001$).

peroxide). After incubation (5 min, 25 °C) the reaction was stopped with 100 μ L of hydrochloric acid (1.2 M). Finally, the UV absorbance of samples was measured by a spectrophotometer (Ultrospec 2000[®]UV; Uppsala Sweden) at $\lambda = 400$ nm (Liu *et al.* 1999).

Liver lipid peroxidation (LPO)

Thiobarbituric acid reactive substances (TBARS) were measured as an index of liver LPO (Uchiyama and Mihara 1978). Briefly, 500 μ L of 10% liver homogenate (in KCl 1.15% w/v) was added to a reaction mixture consists of 3 mL of metaphosphoric acid (1%, w/v) and 1 mL of thiobarbituric acid (1%, w/v) and vigorously vortexed. Afterward, samples were heated (100 °C; 45 minutes). Finally, 4 mL of n-butanol was added and mixed. The resultant mixture was centrifuged (765 g for 5 minutes), and the absorbance of developed color in n-butanol phase was read at $\lambda = 532$ nm using an Ultrospec 2000[®]UV spectrophotometer (Uppsala Sweden) (Heidari *et al.* 2014b).

Statistical analysis

Data are shown as a Mean $\pm$ SEM. A one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as the *post*

hoc test was used to evaluate the significance of differences between mean values. A $p \leq 0.05$ was considered statistically significant.

Results

The current investigation was aimed to evaluate if bacterial endotoxin-induced modest inflammation interacts with PTU and/or MMI-induced liver injury. No significant changes in plasma biomarkers of liver injury were detected when animals were treated with MMI (10, 25 and 50 mg/kg) (Figures 1 and 2). PTU (10, 25 and 50 mg/kg) administration also caused no significant changes in plasma ALT, LDH and AST level as compared with vehicle-treated animals (Figures 1 and 2). A non-injurious dose of LPS (100 μ g/kg, i.p) was administered to mice. Afterward, LPS-treated animals received MMI (10, 25, and 50 mg/kg) and/or PTU (10, 25 and 50 mg/kg) (Figures 1 and 2). It was found that MMI and PTU caused a significant and drastic elevation in plasma level of ALT, LDH, and AST when antithyroid drugs were administered to LPS-treated animals (Figure 1 and 2). On the other hand, plasma TNF- α was significantly increased in LPS and drug cotreated animals

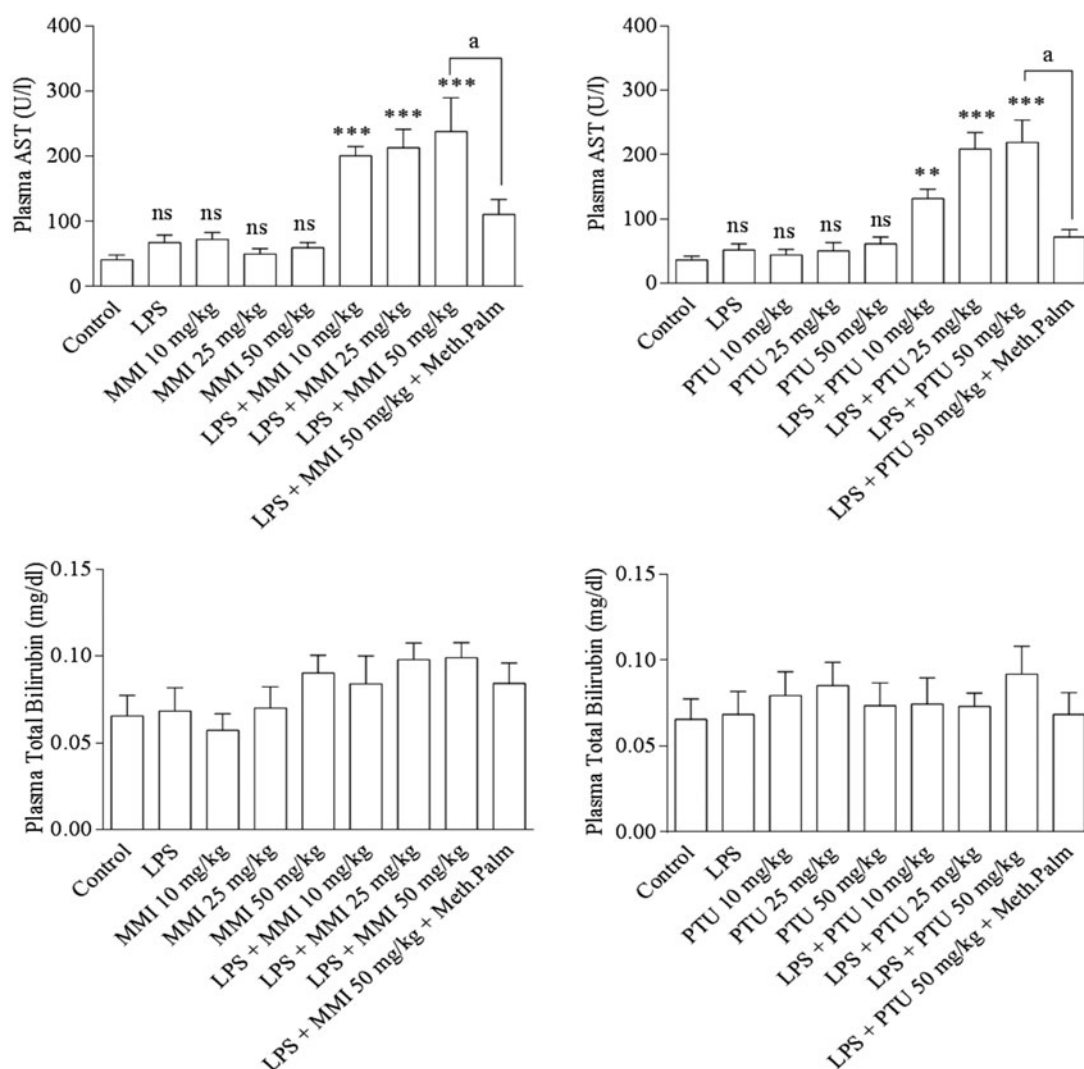


Figure 2. Plasma AST and bilirubin levels in MMI and PTU-treated animals. Data are given as Mean $\pm$ SEM ($n = 5$). MMI: MMI; PTU: propylthiouracil; LPS: lipopolysaccharide; Meth.Palm: Methyl palmitate. LPS (100 μ g/kg, i.p) was administered 2 hours before MMI and/or PTU. Methyl palmitate (2 g/kg, i.v) was administered 72 hours before LPS treatment. ns: not significant as compared to control group. **Indicates significantly different as compared to control animals ($p < 0.01$). ***Indicates significantly different values as compared to control animals ($p < 0.001$). ^aIndicates significantly different values as compared to methyl palmitate-treated groups ($p < 0.001$).

(Figure 3). No significant changes in plasma bilirubin level were detected (Figure 2).

No significant changes in liver GSH content and LPO were detected when animals received MMI (10, 25 and 50 mg/kg) and PTU (10, 25 and 50 mg/kg) (Table 1). On the other hand, when animals were pretreated with LPS, a significant hepatic GSH depletion and an increase in liver tissue level of LPO were found after MMI and/or PTU administration (Table 1).

No significant histopathological changes were detected in the liver of animals received only MMI and/or PTU at the mentioned doses (Table 2). On the other hand, severe histopathological changes including inflammatory cell infiltration (hepatitis), sinusoidal congestion, endotheliitis, liver fibrosis and fatty changes (steatosis) were evident when MMI (10, 25 and 50 mg/kg) and/or PTU (10, 25 and 50 mg/kg) were administered to LPS pretreated animals (Table 2) (Figure 4).

It was found that MPO enzyme activity was significantly elevated in LPS-treated animals (Figure 5). Moreover, MPO enzyme activity was higher in the liver of animals received both LPS and antithyroid drugs (Figure 5).

It was investigated if liver macrophage (Kupffer cells) inhibition alleviated antithyroids hepatotoxicity in LPS-treated animals. It was found that methyl palmitate (2 g/kg, i.v, 44 hours before LPS administration) (Rizzardini *et al.* 1998), prevented MMI and PTU-induced elevation in plasma ALT, LDH and AST (Figures 1 and 2). Furthermore, increased LPO level and liver GSH depletion were mitigated in methyl palmitate-treated animals (Table 1). However, there were no significant changes in liver hepatitis in methyl palmitate and drug-treated animals in the current investigation (Table 2). No significant changes in plasma TNF- α levels were detected in methyl palmitate-treated animals (Figure 3). Moreover, no significant change in MPO activity was detected when animals received methyl palmitate (Figure 5).

It is noteworthy to mention that some data for representing significant changes in oxidative stress markers such as liver tissue LPO and glutathione content might be similar in Table 1 and a previously published manuscript in the field of drug-inflammation interaction by our research team (Niknahad *et al.* 2016).

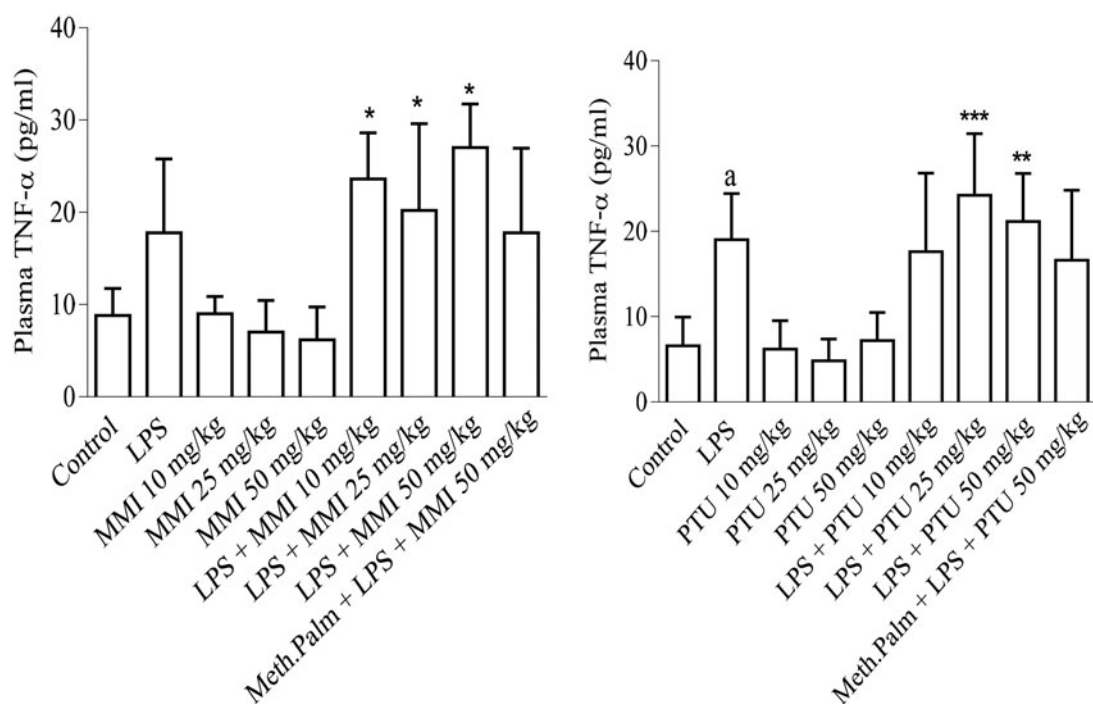


Figure 3. Plasma TNF- α levels in different experimental groups. MMI: MMI; PTU: propylthiouracil; LPS: lipopolysaccharide; Meth.Palm: Methyl palmitate. LPS (100 μ g/kg, i.p) was administered 2 hours before MMI and/or PTU. Methyl palmitate (2 g/kg, i.v) was administered 72 hours before LPS treatment. Data are given as Mean $\pm$ SEM for five animals in each group. *Indicates significantly different values as compared to only drug-treated animals ($p < 0.05$). **Indicates significantly different values as compared to only drug-treated animals ($p < 0.01$). ***Shows significantly different values as compared to only drug-treated animals ($p < 0.001$). ^aIndicates significantly different values as compared to control animals ($p < 0.01$).

Table 1. Hepatic lipid peroxidation and glutathione content in different experimental groups.

Treatment	Liver GSH (μ mol/mg liver tissue)	Lipid peroxidation (nmol of TBARS/mg liver tissue)
Control	43.5 $\pm$ 2.3	2.3 $\pm$ 0.20
Meth.Palm 2 g/kg	49 $\pm$ 4.5	2.7 $\pm$ 0.13
MMI 10 mg/kg	34.8 $\pm$ 3.6	2.9 $\pm$ 0.40
MMI 25 mg/kg	50 $\pm$ 3.5	2.1 $\pm$ 0.30
MMI 50 mg/kg	37 $\pm$ 1.3	2.5 $\pm$ 0.10
PTU 10 mg/kg	44.1 $\pm$ 3.1	2.6 $\pm$ 0.30
PTU 25 mg/kg	46.4 $\pm$ 4.6	3.5 $\pm$ 0.20
PTU 50 mg/kg	40 $\pm$ 4.3	3.3 $\pm$ 0.50
LPS	43.8 $\pm$ 2.7	3.2 $\pm$ 0.26
LPS + MMI 10 mg/kg	28.5 $\pm$ 1.2*	7.2 $\pm$ 1.00*
LPS + MMI 25 mg/kg	22.6 $\pm$ 1.4*	10 $\pm$ 1.50*
LPS + MMI 50 mg/kg	16.4 $\pm$ 4.3*	8.6 $\pm$ 1.1*
Meth.Palm 2 g/kg + LPS + MMI 50 mg/kg	37.3 $\pm$ 2.8**	3.9 $\pm$ 0.14**
LPS + PTU 10 mg/kg	22.2 $\pm$ 0.73*	5.5 $\pm$ 0.20*
LPS + PTU 25 mg/kg	15.5 $\pm$ 2.7*	6.4 $\pm$ 0.50*
LPS + PTU 50 mg/kg	11.7 $\pm$ 1.7*	10.2 $\pm$ 0.40*
Meth.Palm 2 g/kg + LPS + PTU 50 mg/kg	32 $\pm$ 2**	6.34 $\pm$ 0.40**

Data are shown as Mean $\pm$ SEM for five animals in each group.

LPS: lipopolysaccharide; Meth.Palm: methyl palmitate; MMI: MMI; PTU: propylthiouracil; LPS (100 μ g/kg, i.p) was administered 2 hours before MMI and/or PTU. Methyl palmitate (2 g/kg, i.v) was administered 72 hours before LPS treatment.

*Indicates significantly different values as compared to LPS-treated animals ($p < 0.001$).

**Indicates significantly different as compared with LPS + PTU/MMI 50 mg/kg group ($p < 0.001$).

Discussion

Antithyroids hepatotoxicity is a serious clinical complication especially in pediatrics (Lee *et al.* 2000, Malozowski and Chiesa 2010, Akmal and Kung 2014). Although there is no precise mechanism for antithyroid drug-induced liver injury,

but oxidative stress, drug reactive metabolites and intracellular organelle dysfunction are speculated to be involved in this complication (Mizutani *et al.* 1999, 2000, Heidari *et al.* 2012a, 2012b, 2014c, 2015a). On the other hand, there is a body of evidence on the role of inflammatory cells and cytokines in drug-induced liver injury (Deng *et al.* 2009, Zou *et al.* 2012, Webb and Adams 2015). Several investigations indicate that hepatotoxicity of some xenobiotics was augmented and the threshold for toxicity from these chemicals was lowered by co-exposure to LPS (Barton *et al.* 2000, Luyendyk *et al.* 2003, Deng *et al.* 2006, Karaa *et al.* 2008, Bachour-El Azzi *et al.* 2014, Su *et al.* 2014). Previously we found that the hepatotoxic profile of antimalarial drugs-induced was deteriorated in the presence of a mild inflammatory response (Niknahad *et al.* 2016). On the other hand, the interaction between inflammatory response and liver injury induced by several classes of drugs have been proved (Barton *et al.* 2000, Luyendyk *et al.* 2003, Deng *et al.* 2006, Karaa *et al.* 2008, Bachour-El Azzi *et al.* 2014, Su *et al.* 2014). All these data mention an important potential interaction between inflammatory responses and drug-induced liver injury. Moreover, these investigations mention the 'drug-inflammation interaction model' as a valuable experimental tool to study the mechanisms of drug-induced liver injury. The current investigation was designed to evaluate if the antithyroid drugs-induced liver injury is augmented by inflammation. We also evaluated the role of kupffer cells inhibition in this process.

In concert with previous investigations (Barton *et al.* 2000, Luyendyk *et al.* 2003, Deng *et al.* 2006, Karaa *et al.* 2008, Bachour-El Azzi *et al.* 2014, Su *et al.* 2014), we found that co-treatment of mice with antithyroid drugs and bacterial endotoxin, which induced a modest inflammation, lead to acute

Table 2. Histopathological changes of mice liver in LPS and antithyroid drugs-treated groups.

Treatment	Hepatitis	Fibrosis	Fatty changes	Congestion	Necrosis	Total grade
Control	–	–	–	–	–	0 (Normal liver)
LPS-treated	+	–	–	+	–	2
Methyl palmitate 2g/kg	–	–	–	–	–	0
MMI 10 mg/kg	–	–	–	+	–	1
MMI 25 mg/kg	–	–	–	++	–	2
MMI 50 mg/kg	–	–	–	+	–	1
PTU 10 mg/kg	–	–	–	++	–	2
PTU 25 mg/kg	–	–	–	++	–	1
PTU 50 mg/kg	–	–	–	++	–	2
LPS + MMI 50 mg/kg	++++	–	–	+	–	5
LPS + PTU 50 mg/kg	+++	+	+	++	–	7
LPS + MMI 50 mg/kg + Methyl palmitate 2g/kg	++	–	–	+	–	3
LPS + PTU 50 mg/kg + Methyl palmitate 2g/kg	++	–	–	++	–	4

LPS: lipopolysaccharide; PTU: propylthiouracil.

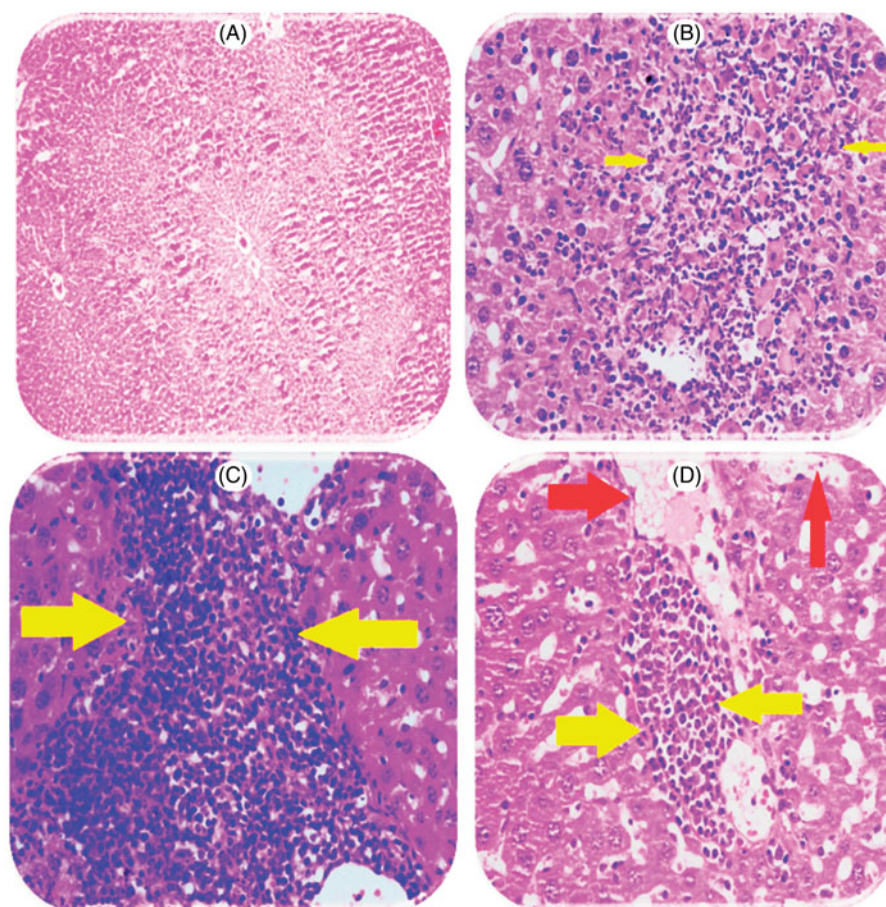


Figure 4. Photomicrographs of liver histopathological changes in lipopolysaccharide and antithyroid drugs-treated groups. (A) Control mice liver; showed no significant histopathological lesions. (B) LPS-treated mice; inflammatory cells (yellow arrow) was detected in this group. (C) LPS + MMI (50 mg/kg); massive inflammatory cells aggregation and hepatitis were found in this group (Yellow arrows). (D) LPS + PTU (50 mg/kg); inflammatory cells infiltration and hepatitis (Yellow arrows), in addition to sinusoidal congestion (Red arrow) are evident in this group.

liver injury, whereas neither LPS nor antithyroid drugs were hepatotoxic alone. Plasma biomarkers of liver injury were significantly increased in LPS and antithyroid drugs co-treated animals in the current investigation, which indicates antithyroid drugs hepatotoxicity.

Inflammatory stress might sensitize the liver to injury (Roth *et al.* 2003). On the other hand, episodic inflammations are commonplace in patients (Roth *et al.* 2003). Therefore, we might be able to suggest that inflammatory stresses occurring during antithyroid drugs administration could contribute to drug-induced liver injury and enhance the sensitivity of the liver to the toxic effects of these drugs.

Endotoxin itself is not hepatotoxic at low concentrations (100 µg/kg, i.p) (Mignon *et al.* 1999, Luyendyk *et al.* 2004). However, its ability to stimulate an inflammatory response is involved in its pathogenicity in the liver. LPS directly stimulates toll-like receptors (TLRs) and activates Kupffer cells (Su 2002) (Figure 5). These cells may be involved in the destruction of hepatocytes by producing harmful mediators, such as inflammatory cytokines and proteolytic enzymes, and chemo-attraction of other inflammatory cells to the liver tissue (Kolios *et al.* 2006, Dixon *et al.* 2013). Interestingly, a recent study by Akai *et al.* indicates that kupffer cells are involved in MMI-induced liver injury (Akai *et al.* 2015). This

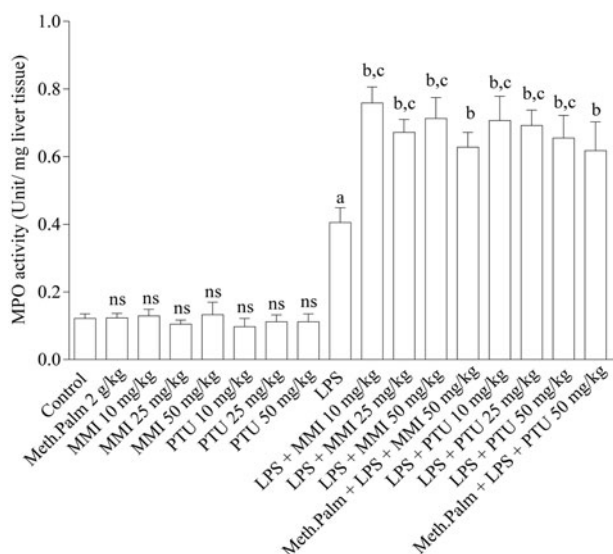


Figure 5. Myeloperoxidase (MPO) activity in the liver tissue of LPS and antithyroid drugs-treated animals. MMI: MMI; PTU: propylthiouracil; LPS: lipopolysaccharide; Meth.Palm: Methyl palmitate. LPS (100 μ g/kg, i.p) was administered 2 hours before MMI and/or PTU. Methyl palmitate (2 g/kg, i.v) was administered 72 hours before LPS treatment. Data are given as Mean $\pm$ SEM for five animals. ns: not significant as compared to control animals. ^aIndicates significantly different values as compared to control animals ($p < 0.01$). ^bShows significantly different values as compared to control animals ($p < 0.001$). ^cSignificantly different as compared to LPS-treated group ($p < 0.05$).

investigation indicates that kupffer cells are responsible for MMI-induced liver injury in a glutathione-depleted rat model. It has been found that inactivation of kupffer cells blunted MMI-induced hepatic damage in glutathione-depleted animals (Akai *et al.* 2015). In this study, we found that Kupffer cells inhibition by methyl palmitate alleviated not only MMI hepatotoxicity but also PTU-induced liver injury in LPS-treated animals. These findings indicate that Kupffer cells could play a major role in PTU-induced hepatotoxicity. However, methyl palmitate administration caused no significant changes in plasma TNF- α level or tissue MPO activity (Figures 3 and 6). These data could predict a role for other inflammatory cells such as neutrophils in the situation.

As mentioned, neutrophils and other immune cells might also be responsible for the liver injury induced by MMI and PTU (Figure 6). Inhibition of these cells (e.g. macrophage inhibition by methyl palmitate) might prevent drug bioactivation, at least in part, and consequently prevent liver injury (Figure 6). Hence, the application of more broad acting inflammatory cell inhibitors and antibodies might enhance our understanding of the contribution of other immune cells in antithyroid drug-induced hepatotoxicity. Furthermore, using inbred animals are better choices to judge the role of immune system-mediated mechanisms of injury.

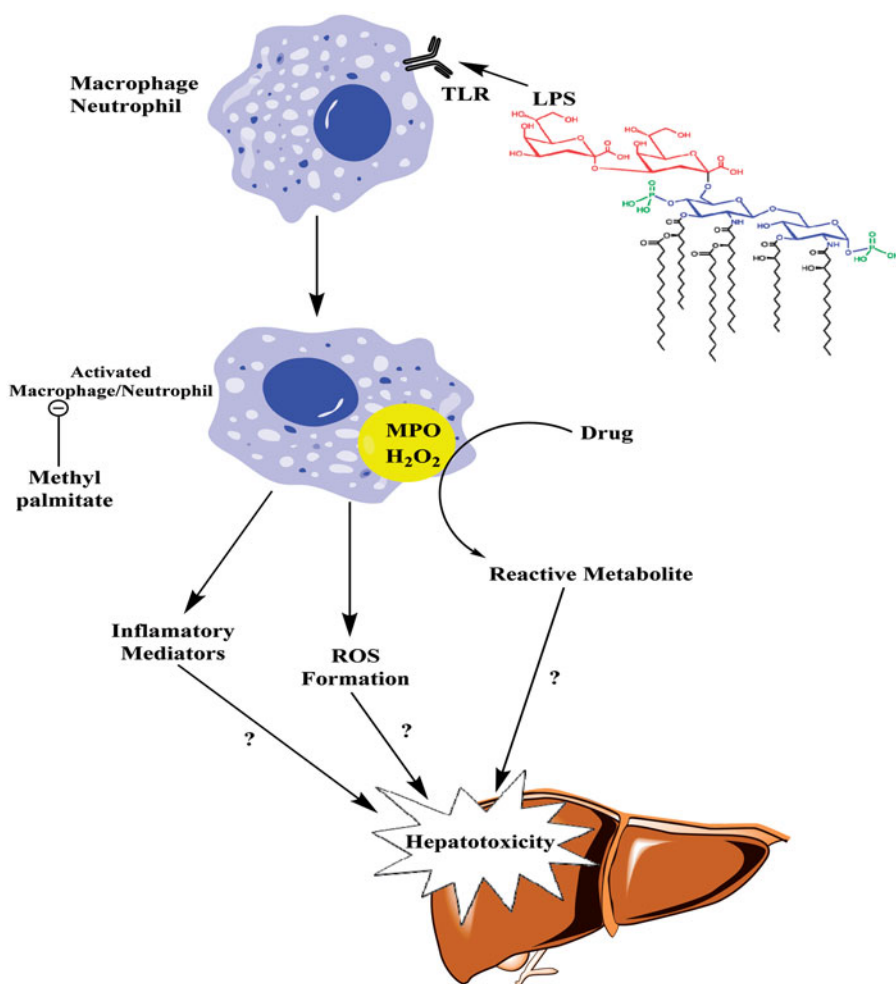


Figure 6. The proposed mechanisms for exacerbation of hepatotoxicity induced by antithyroid drugs in drug-inflammation interaction model. LPS: Lipopolysaccharide; TLR: Toll-like receptor; ROS: Reactive oxygen species; MPO: Myeloperoxidase.

Although many parameters of the inflammatory response contribute to liver injury (Moulin *et al.* 2001), one well-studied pathway is the production of cytotoxic cytokines such as TNF- α . In many models of liver injury elevated TNF- α levels are present and correlated with xenobiotics-induced hepatotoxicity (Holt and Ju 2006). In the current study, we found that plasma TNF- α level was significantly increased in LPS-treated animals (Figure 3). Other cytokines such as IL-1 β , and INF- γ , produced by other inflammatory cells (e.g. natural killers, T-helpers), might also be involved in the liver injury in drug-inflammation interaction model (Figure 6). Interestingly, some investigations mentioned MMI-induced cytokine release from T-cells (IL-4) as a mechanism to induce hepatocyte injury (Kobayashi *et al.* 2012). There is no report of PTU-induced cytokine release from immune cells so far. In the current investigation, we found that serum TNF- α level was significantly higher in antithyroid drugs-treated animals that mention a role for cytokines in antithyroid drugs-induced hepatotoxicity.

Inflammation and oxidative stress are firmly interconnected events (Chaudhari *et al.* 2014). It has been well established that activated macrophages are the main source of reactive oxygen species (ROS) (Figure 6) (Mittal *et al.* 2014). In the current study, we found that when mice were pretreated with LPS, significant hepatic GSH depletion and an increase in liver tissue LPO level were found after MMI and/or PTU administration (Table 1). Hence, oxidative stress could play an important role in antithyroid drugs-induced liver injury in endotoxin-treated animals.

Several cases of antithyroid drugs-induced liver failure are reported in patients with an infectious liver disease background (e.g. Hepatitis) (Kang *et al.* 1990, Gupta and Lewis, 2008). This might indicate the clinical relevance of inflammation and inflammatory cells in antithyroid drugs-induced liver injury in patients.

MPO enzyme is present in neutrophils, tissue macrophages and monocytes. Assessment of liver MPO activity in this study (Figure 5), indicated accumulation of inflammatory cells including neutrophils in liver tissue (Figures 4 and 5). The role of MPO in drug metabolism and its relevance to drug-induced injury has been mentioned in previous investigations (Eghbal *et al.* 2004, Tafazoli and O'brien 2005, Macallister *et al.* 2013). Interestingly, it has been found that MPO-mediated metabolism of MMI is responsible for oxidative stress and glutathione depletion in an *in vitro* system (McGirr *et al.* 1990). Moreover, it has been reported that PTU is metabolized by neutrophils MPO in blood, a metabolic pathway which might be associated with agranulocytosis as a dangerous side effect of this drug (Waldhauser and Uetrecht 1991). Hence, reactive metabolites produced in the inflamed liver might contribute to MMI/PTU-induced liver injury in our study.

Collectively, data presented in the current investigation indicate a synergistic liver injury from antithyroid drugs and bacterial lipopolysaccharide coexposure. Indeed, evaluating the effect of inflammation and inflammatory cytokines on antithyroid drugs-induced liver injury in other experimental models, as well as in a wider period will provide new insights for the pathological pattern of liver injury and the precise mechanism of toxicity induced by these drugs (Figure 6). Moreover, these data might lead to new therapeutic

strategies against drug-induced liver injury and developing animal and/or *in vitro* models, which predict the potential hepatotoxic properties of xenobiotics.

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