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Research Article

Concurrent Inflammation Augments Antimalarial Drugs-Induced Liver Injury in Rats

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Abstract

Purpose: Accumulating evidence suggests that drug exposure during a modest inflammation induced by bacterial lipopolysaccharide (LPS) might increase the risk of drug-induced liver injury. The current investigation was designed to test if antimalarial drugs hepatotoxicity is augmented in LPS-treated animals.

Methods: Rats were pre-treated with LPS (100 µg/kg, i.p). Afterward, non-hepatotoxic doses of amodiaquine (25, 50 and 100 mg/kg, oral) and chloroquine (25, 50 and 100 mg/kg, oral) were administered.

Results: Interestingly, liver injury was evident only in animals treated with both drug and LPS as estimated by pathological changes in serum biochemistry (ALT, AST, LDH, and TNF-α), and liver tissue (severe hepatitis, endotheliitis, and sinusoidal congestion). An increase in liver myeloperoxidase enzyme activity, lipid peroxidation, and protein carbonylation, along with tissue glutathione depletion were also detected in LPS and drug co-treated animals.

Conclusion: Antimalarial drugs rendered hepatotoxic in animals undergoing a modest inflammation. These results indicate a synergistic liver injury from co-exposure to antimalarial drugs and inflammation.

Introduction

Chloroquine and amodiaquine are widely administered against malaria.[1] Chloroquine is also used in the management of lupus erythematosus and rheumatoid arthritis.[2,3] Chloroquine is one of the most effective disease-modifying antirheumatic drugs (DMARDs).[5] Amodiaquine is used in the prophylaxis and treatment of malaria, especially against chloroquine-resistant isolates of *Plasmodium falciparum*.[4,5] Although safe and approximately non-hepatotoxic agents such as doxycycline are available for malaria treatment, but a range of adverse effects including hepatotoxicity is attributed to malaria drug therapy with chloroquine and amodiaquine.[6-10] Several cases of antimalarial drugs-induced liver injury are reported, but there is no clear mechanism for antimalarial drugs hepatotoxicity. Some investigations indicated the role of reactive metabolites and oxidative stress in chloroquine-induced liver injury.[11-14] Bioactivation of amodiaquine to a quinone imine metabolite,[15] and oxidative stress have also been suggested to be involved in the development of amodiaquine-induced hepatotoxicity.[16,17]

Drug-inflammation interaction is an intriguing model for investigating the mechanisms of drug-induced liver injury.[18,19] It has been reported that a modest and non-injurious inflammation interacts with a small dose of drugs and induces hepatotoxicity.[20,21] Bacterial lipopolysaccharide (LPS, Endotoxin) is widely applied in this model to induce a modest inflammation.[20] The hepatotoxicity of many xenobiotics has been augmented in LPS-treated animals.[22-24]

On the other side, malaria infection is associated with an inflammatory response in many tissues including liver.[25-27] Immune cells are aggregated in the liver of malaria-infected patients.[25-27] This might interact with malaria drug therapy and finally, leads to drug-induced liver injury. The current investigation was designed to evaluate if a modest inflammation interacts with non-hepatotoxic doses of antimalarial drugs to induce liver injury in rats.

Materials and Methods

Chemicals

Amodiaquine, 5, 5´-dithiol nitrobenzoic acid (DTNB), Ortho-dianisidine hydrochloride, Hexadecyl trimethyl ammonium bromide (HTAB), Chloroquine, Triton X-100, 2,4-dinitrophenyl hydrazine (DNPH), Guanidine

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hydrochloride, and n-Butanol were purchased from Sigma-Aldrich (St. Louis, USA). Methyl palmitate, Ethylenediaminetetraacetic acid (EDTA), Trichloroacetic acid (TCA,) Thiobarbituric acid (TBA), Hydrogen peroxide (H_2O_2), Ethanol, Methanol, Ethyl acetate, and Hydroxymethyl amino methane-Hydrochloride (Tris-HCl), were purchased from Merck (Dardamstd, Germany). Bacterial lipopolysaccharide (LPS) from *E.coli* was prepared from Iranian Biological Resource Center (IBRC) (Tehran, Iran). Kits for liver biochemistry analysis (ALT, LDH, AST, and bilirubin) were obtained from Pars Azmun ® Company (Tehran, Iran). TNF-α kit was purchased from eBioscience. All salts for preparing buffer solutions were of the highest grade commercially available and prepared from Merck (Dardamstd, Germany).

Animals

Male Sprague-Dawley rats (200-250 g weight, n=85), were obtained from laboratory animals breeding center, Shiraz University of Medical Sciences (Shiraz, Iran). Animals were housed in cages on wood bedding at an ambient temperature of 23±1 °C and had access to food and water *ad libitum*. Animals received humane care and use and were handled according to the animal handling protocol at Shiraz University of Medical Sciences, approved by a local ethics committee (#93-7076).

Experimental setup

Rats were randomly divided equally into 17 groups (n=5): **A**) Control (vehicle-treated); **B**) LPS (100 µg/kg, i.p); **C**) Amodiaquine (25 mg/kg, gavage); **D**) Amodiaquine (50 mg/kg, gavage); **E**) Amodiaquine (100 mg/kg, gavage); **F**) Chloroquine (25 mg/kg, gavage); **G**) Chloroquine (50 mg/kg, gavage); **H**) Chloroquine (100 mg/kg, gavage); **I**) LPS (100 µg/kg, i.p) + Amodiaquine (25 mg/kg, gavage); **J**) LPS (100 µg/kg, i.p) + Amodiaquine (50 mg/kg, gavage); **K**) LPS (100 µg/kg, i.p) + Amodiaquine (100 mg/kg, gavage); **L**) Methyl palmitate (2g/kg, i.v); **M**) Methyl palmitate (2g/kg, i.v) + LPS (100 µg/kg) + Amodiaquine (100 mg/kg, gavage); **N**) LPS (100 µg/kg) + Chloroquine (25 mg/kg, gavage); **O**) LPS (100 µg/kg) + Chloroquine (50 mg/kg, gavage); **P**) LPS (100 µg/kg) + Chloroquine (100 mg/kg, gavage); **Q**) Methyl palmitate (2g/kg, i.v) + LPS (100 µg/kg, i.p) + Chloroquine (100 mg/kg, gavage).
Bacterial LPS (100 µg/kg, i.p),[28,29] was administered 2 hours before antimalarial drugs. Methyl palmitate (2 g/kg, i.v) was applied as an effective kupffer cells inhibitor and administered 44 hours before LPS.[30] Liver injury biomarkers were assessed 24 hours after antimalarial drugs administration in all experimental groups.

Serum biochemistry and liver histopathology

Animals were anesthetized (pentobarbital, 50 mg/kg, i.p) and blood samples were collected from the abdominal *vena cava*. The liver was removed and washed in cooled-sodium chloride 0.9% (w/v). Serum samples were prepared by centrifugation (3000 *g*, 15 min, 4°C) and stored at -70°C for further analysis of ALT, LDH, AST, bilirubin, and TNF-α. For tissue histopathological evaluation, samples of liver were fixed in neutral-buffered formalin solution (0.4% sodium phosphate monobasic, NaH_2PO_4, 0.64% sodium phosphate dibasic, Na_2HPO_4, and 10% formaldehyde in distilled water).[31] Paraffin-embedded sections of liver were prepared and stained with hematoxylin and eosin (H&E) before light microscope viewing.

Liver glutathione content

Liver tissue glutathione content was assessed by the Ellman reagent (DTNB).[32] Briefly, liver samples (200 mg) were homogenized in 8 ml of EDTA solution (0.02 M, 4°C). Then, 5 mL of liver homogenate was mixed with 4 mL of distilled water (4°C), and 1 mL of trichloroacetic acid (50%, w/v). The mixture was vortexed and centrifuged (765 *g*, 15 minutes, 4°C). Afterward, 2 mL of supernatant was added to 4 mL of Tris buffer (pH= 8.9) and 100 µl of DTNB solution (0.01 M in methanol).[32,33] Finally, the samples absorbance was assessed at 412 nm using an Ultrospec 2000®UV spectrophotometer.

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured as an index of lipid peroxidation in liver tissue.[34] Briefly, the reaction mixture was consisted of 0.5 mL of 10% liver homogenate, 3 mL of metaphosphoric acid (1%, w/v) and 1 mL of thiobarbituric acid (1% , w/v). The mixture was heated (100°C, 45 minutes). Then, 4 mL of n-butanol was added to the reaction mixture and vigorously mixed. After centrifugation in 765*g* for 5 minutes, the absorbance of developed color in n-butanol phase was measured at 532 nm using an Ultrospec 2000®UV spectrophotometer.[35]

Protein carbonylation in liver tissue

The oxidative damage of proteins was assessed by dinitrophenylhydrazine (DNPH) method.[36] Briefly, a 10% liver homogenate (w/v) was prepared in phosphate buffer solution (pH 7.5) containing 0.1% Triton X-100. The homogenate was centrifuged (700 g, 10 min, 4°C). Then, 500 µl aliquots of the resulting supernatant were added to 300 µl of 2, 4-dinitrophenylhydrazine (DNPH) (10 mM, dissolved in 2 M HCl). The samples were incubated in dark with vortexing every 10 minutes (1 hour, 25°C). Then, 100 µL of trichloroacetic acid (20% w/v) was added. Tubes were centrifuged at 11,000 rpm for 3 minutes, and the supernatant discarded. The pellet was washed 3 times with 1 ml ethanol: ethyl acetate (1:1 v: v). Samples were left 10 minutes before centrifugation and the supernatant was discarded each time. The precipitate was redissolved in 600 µl of guanidine solution (8 M in 20 mM potassium phosphate, pH=2.3) and incubated for 15 minutes at 37°C. Finally, the solution was centrifuged (11,000 rpm for 3 min) and its absorbance was determined at 370 nm using an

Ultrospec 2000®UV spectrophotometer (Uppsala, Sweden).[37,38]

Myeloperoxidase enzyme (MPO) activity in liver tissue

MPO activity was assessed as an index of tissue inflammatory cells accumulation. To measure liver tissue MPO activity, 500 mg of tissue was homogenized in a solution containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB) dissolved in potassium phosphate buffer (50 mM, pH=6) and then centrifuged (3000 g, 20 min, 4°C). Afterward, 100 µl of the supernatant was added to 2.9 mL of potassium phosphate buffer solution (50 mM, pH=6) containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.0005% H_2O_2. After 5 min of incubation, the reaction was stopped with 0.1 ml of 1.2 M hydrochloric acid. The rate of change in absorbance was measured by a spectrophotometer (Ultrospec 2000®UV) at 400 nm. Myeloperoxidase activity was expressed in units per 100 mg weight of wet liver tissue.[39]

Statistical analysis

The results are expressed as a Mean±SEM. A one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as a *post hoc* test was used to assess the significance of differences between mean values. A $p<0.05$ was considered to be statistically significant.

Results

The current investigation was designed to examine the effects of bacterial endotoxin (LPS) on amodiaquine (AQ) and/or chloroquine (CQ)-induced liver injury. It was found that AQ (25, 50, and 100 mg/kg, oral) caused no significant changes in serum level of liver injury biomarkers when it was administered alone (Figure 1). CQ administration (25, 50, and 100 mg/kg, oral) also caused no significant elevation in ALT, LDH, AST, and serum total bilirubin as compared to control animals (Figure 2).

Rats were pre-treated with a non-injurious dose of bacterial endotoxin (100 µg/kg of LPS, i.p). Then, animals received AQ (25, 50, and 100 mg/kg, oral) and/or CQ (25, 50, and 100 mg/kg, oral) (Figure 1). Interestingly, it was found that AQ and CQ caused a significant and severe elevation in serum ALT, LDH, and AST level when these drugs were administered to LPS-treated animals (Figure 1 and 2). No significant changes in serum bilirubin levels were identified between different groups (Figure 3). A significant elevation in serum TNF-α was detected in LPS and drug-treated animals (Figure 4).

It was found that the sole administration of AQ (25, 50, and 100 mg/kg, oral) and/or CQ (25, 50, and 100 mg/kg, oral) caused no significant changes in liver glutathione content, lipid peroxidation, and protein carbonylation (Table 1). When animals were pre-treated with LPS (100 µg/kg, i.p), significant increase in lipid peroxidation and protein carbonylation, along with liver tissue glutathione depletion were detected after AQ (25, 50, and 100 mg/kg, oral) and/or CQ (25, 50, and 100 mg/kg, oral) administration (Table 1).

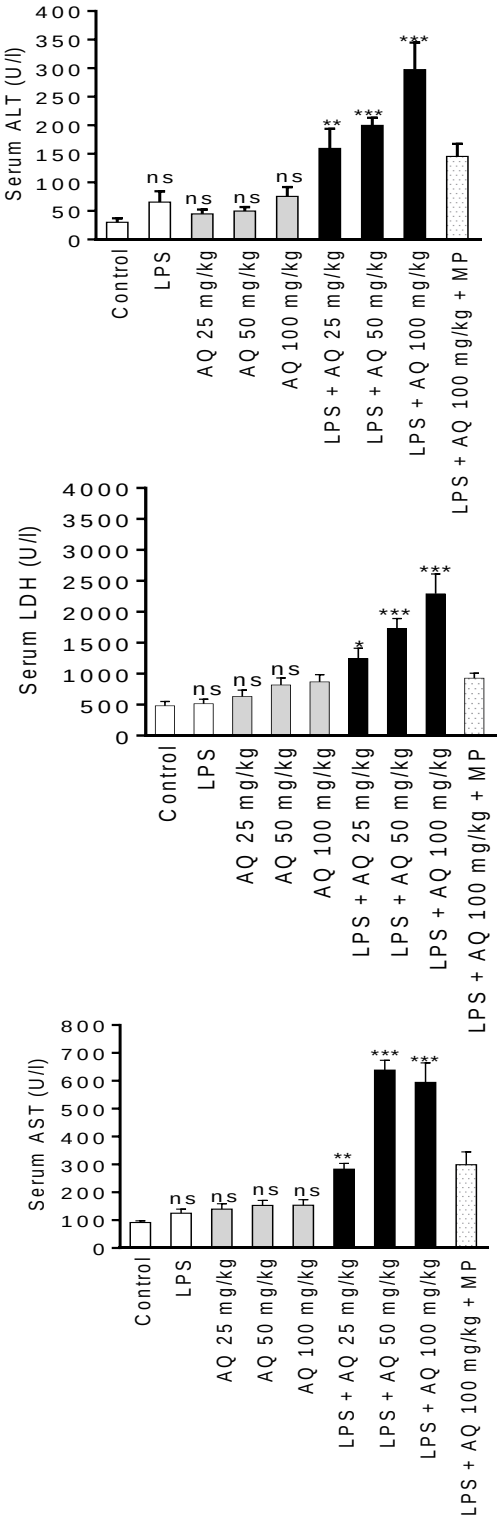

Figure 1. Serum biomarkers of liver injury in amodiaquine (AQ)-treated animals.
LPS: lipopolysaccharide; AQ: Amodiaquine; MP: Methyl palmitate.
Data are given as Mean±SEM for five animals in each group.
Asterisks indicate significantly different as compared with LPS-treated group (*=P<0.05; **=P<0.001; ***=P<0.0001).

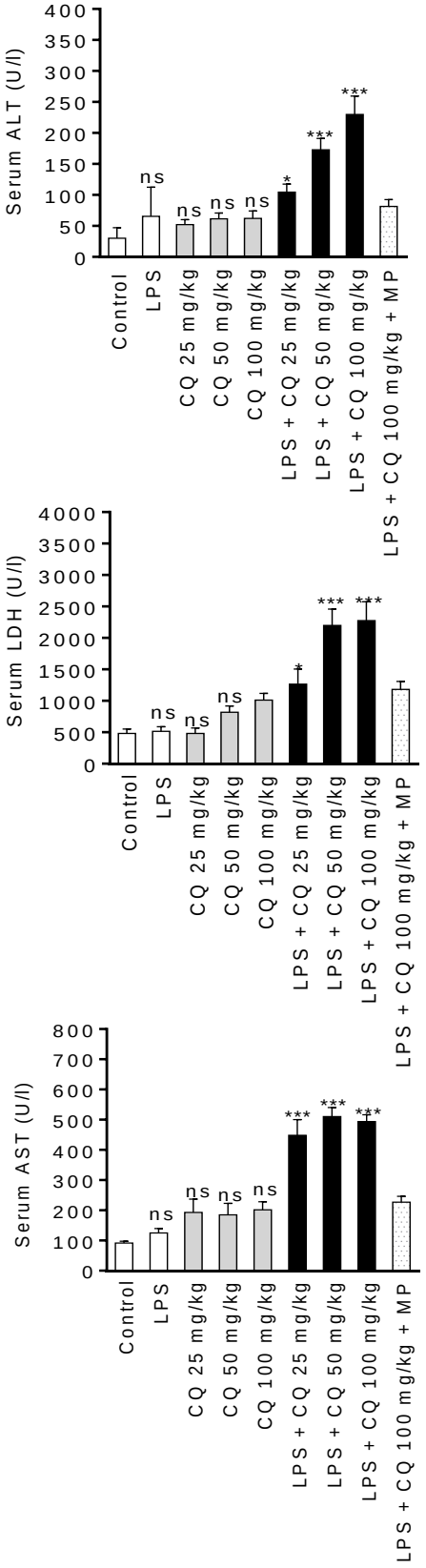

Figure 2. Biomarkers of liver injury in chloroquine (CQ)-treated rats.
LPS: lipopolysaccharide; CQ: Chloroquine; MP: Methyl palmitate.
Data are given as Mean±SEM (n=5).
Asterisks indicate significantly different as compared with LPS-treated group (*=P<0.05; **=P<0.001; ***=P<0.0001).

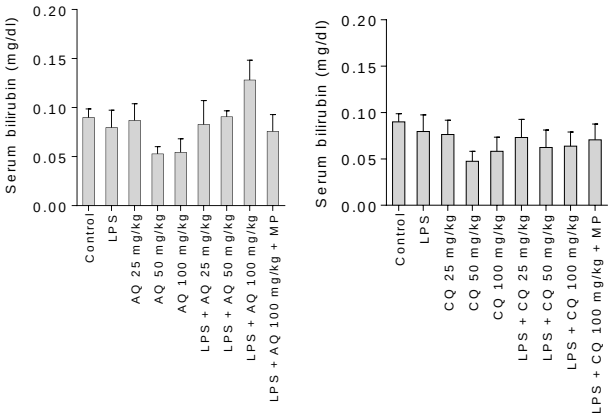

Figure 3. Serum level of total bilirubin. LPS: lipopolysaccharide; CQ: Chloroquine; AQ: Amodiaquine; Meth.Palm: Methyl palmitate. Data are given as Mean±SEM for five animals in each group. No significant changes were detected between serum total bilirubin levels of different experimental groups.

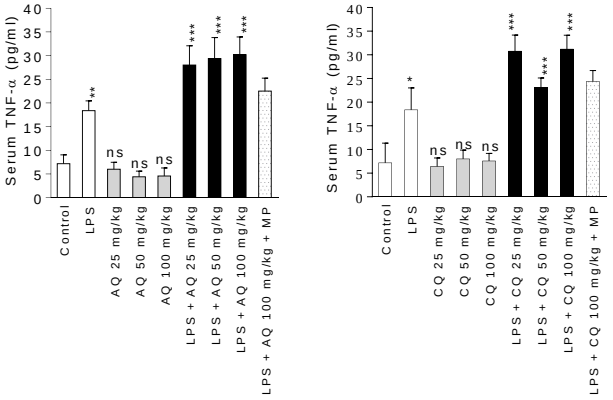

Figure 4. Serum TNF-α levels in LPS and drug-treated animals. LPS: lipopolysaccharide; CQ: Chloroquine; AQ: Amodiaquine; MP: Methyl palmitate. Data are given as Mean±SEM (n=5). ns: not significant as compared with control animals. Asterisks indicate significantly different as compared with control (*=P<0.05; **=P<0.001; ***=P<0.0001).

Histopathological evaluation of liver tissue in AQ (25, 50 and 100 mg/kg) and CQ (25, 50 and 100 mg/kg)-treated animals revealed no significant changes in comparison with control animals (Figure 5). On the other hand, significant inflammatory cell infiltration (hepatitis and endotheliitis), and sinusoidal congestion were detected when LPS-treated animals received AQ (100 mg/kg) and/or CQ (100 mg/kg) (Figure 5).

Assessment of MPO activity in liver tissue revealed a high amount of the enzyme in the liver of LPS-treated rats (Table 1). No significant differences were detected between different LPS and/or drug-treated groups in liver tissue MPO activity (Table 1).

The effect of macrophage (Kupffer cells) inhibition was also investigated in antimalarial drugs and LPS-treated animals. We found that methyl palmitate (2 g/kg, i.v, 44 hours before LPS administration) prevented AQ and CQ-induced elevation in serum level of liver injury biomarkers (Figure 1 and 2), but it had no significant effect on the serum level of TNF-α (Figure 4).

Furthermore, lipid peroxidation, protein carbonylation, and liver glutathione depletion were mitigated in methyl palmitate-treated animals (Table 1).

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

Treatment	*Liver GSH (µmol/mg wet tissue)*	*Lipid peroxidation (nmol of TBARS/mg wet tissue)*	*Protein carbonylation (UV absorption at 370 nm)*	*Myeloperoxidase enzyme activity (Unit/ mg liver tissue)*
Control	44.76±2.30	2.22±0.18	0.101±0.09	0.134±0.08
Meth.Palm 2 g/kg	40.32±1.44	2.05±0.54	0.109±0.08	0.113±0.09
AQ 25 mg/kg	45.22±1.20	3.10±0.20	0.112±0.07	0.102±0.07
AQ 50 mg/kg	40.44±3.50	2.09±0.44	0.134±0.09	0.121±0.09
AQ 100 mg/kg	41.24±2.30	2.87±0.13	0.122±0.10	0.174±0.10
CQ 25 mg/kg	51.21±1.04	2.43±0.35	0.106±0.04	0.139±0.08
CQ 50 mg/kg	44.32±0.53	2.58±0.44	0.131±0.08	0.101±0.09
CQ 100 mg/kg	39.41±2.31	2.05±0.64	0.142±0.17	0.152±0.14
LPS (100 µg/kg, i.p)	48.22±3.25	3.04±0.53	0.357±0.12	0.476±0.19
LPS + AQ 25 mg/kg	31.73±2.27*	6.90±0.91*	0.769±0.11*	0.831±0.12*
LPS + AQ 50 mg/kg	24.14±0.12 *	9.50±1.31*	0.684±0.17*	0.772±0.13*
LPS + AQ 100 mg/kg	19.69±1.32*	8.80±0.95*	0.774±0.10*	0.731±0.11*
Meth.Palm 2 g/kg + LPS + AQ 100 mg/kg	31±2.04	5.31±0.62	0.309±0.11	0.652±0.13
LPS + CQ 25 mg/kg	32.44±2.10*	7.65±1.04 *	0.831±0.18*	0.798±0.12*
LPS + CQ 50 mg/kg	29.31±3.40 *	7.44±0.83*	0.677±0.09*	0.830±0.19*
LPS + CQ 100 mg/kg	24.31±1.40*	8.02±1.23*	0.793±0.12*	0.881±0.16*
Meth.Palm 2 g/kg + LPS + CQ 100 mg/kg	35.21±0.92	4.83±1.09	0.213±0.14	0.606±0.09

Data are shown as Mean±SEM (n=5).
Meth.Palm: Methyl palmitate. AQ: Amodiaquine; CQ: Chloroquine; LPS: lipopolysaccharide. LPS (100 µg/kg, i.p) was administered 2 hours before AQ and/or CQ. Methyl palmitate (2 g/kg, i.v) was administered 44 hours before LPS treatment.
* Indicates significantly different values as compared to LPS-treated animals (P<0.05).

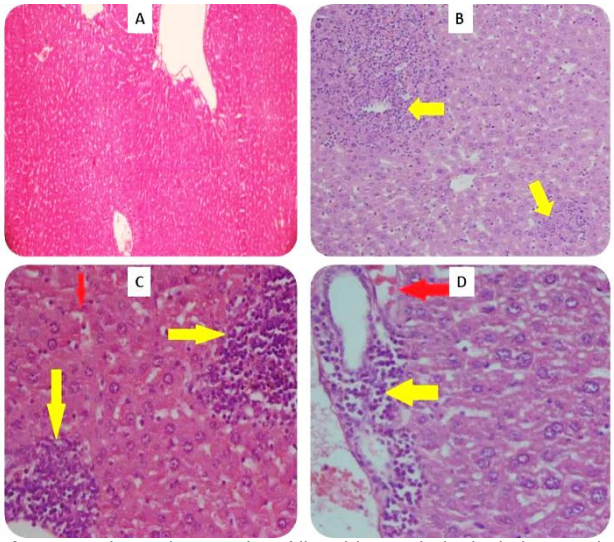

Figure 5. Photomicrographs of liver histopathological changes in LPS and antimalarial drugs-treated animals. **A**: Control rat liver; showed no significant histopathological lesions. **B**: LPS-treated rats; inflammatory cells infiltration (yellow arrow) was detected in this group. **C**: LPS+ Amodiaquine (100 mg/kg); massive inflammatory cells aggregation and hepatitis and sinusoidal congestion (red arrow) were found in this group (Yellow arrows). **D**: LPS+ Chloroquine (100 mg/kg); inflammatory cells infiltration and hepatitis (Yellow arrows), in addition to sinusoidal congestion (Red arrow) are also evident in this group.

It might be noteworthy that the hepatotoxic effect of antimalarial drugs was not dose-dependent in any of the assessed parameters in drug-inflammation interaction model. The comparison between two investigated antimalarial drugs was made based on serum biochemical parameters. No significant differences were found between the severity of liver injury induced by chloroquine and amodiaquine in drug-inflammation interaction model as judged by serum ALT ($P > 0.05$), LDH ($P > 0.05$), and AST ($P > 0.05$). Furthermore, there was also no significant difference in serum TNF-α level when amodiaquine or chloroquine was compared in the LPS-treated animals ($P > 0.05$) (Figure 4).

Discussion

The current investigation was designed to evaluate the effect of concurrent inflammation on the antimalarial drugs-induced liver injury. Several investigations indicated that the threshold for hepatotoxicity from many xenobiotics was lowered by co-exposure to LPS.[22,23,40-43] The ability of LPS to stimulate an inflammatory response may account for its pathogenicity in the liver. LPS is a potent activator of liver tissue macrophages (Kupffer cells) through toll-like receptors (TLRs) (Figure 6).[44] Activated Kupffer cells are a major source of, lysosomal

and proteolytic enzymes in addition of inflammatory mediators including several cytokines, superoxide anion, nitric oxide, eicosanoids, and chemokines.[45-47] Although many parameters of the inflammatory response contribute to liver injury,[48] one well-studied pathway is the production of TNF-α. In many models of hepatotoxicity, the elevated TNF-α level is correlated with liver injury.[49] We found that serum TNF-α level was significantly elevated in LPS-treated animals (Figure 4). Other cytokines rather than TNF-α (*e.g* 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.[23,49,50]

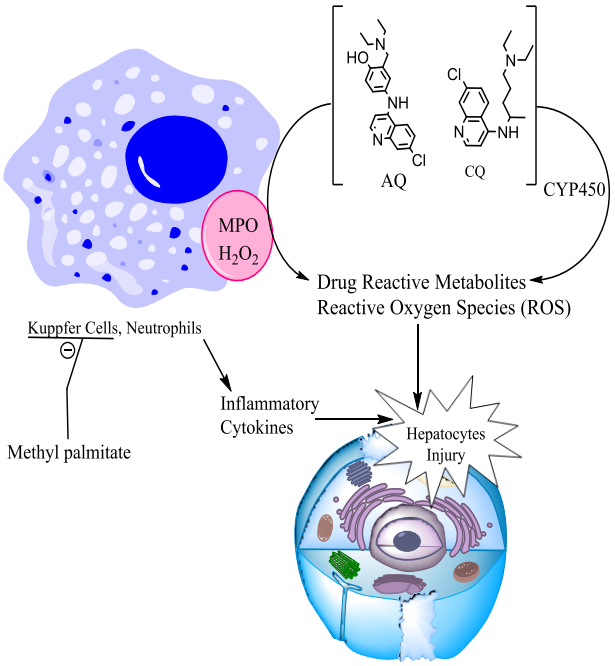

Figure 6. The proposed mechanisms for antimalarial drugs-induced liver injury in LPS-treated animals. AQ: Amodiaquine; CQ: Chloroquine; MPO: Myeloperoxidase.

In the current study, we found that Kupffer cells inhibition by methyl palmitate alleviated antimalarial drugs-induced liver injury in LPS-treated animals. These findings indicate that Kupffer cells might be involved in antimalarial drugs hepatotoxicity. However, methyl palmitate administration caused no significant changes in serum TNF-α level (Figure 4). This might indicate the role of other inflammatory cells such as neutrophils in the situation. It was found that serum TNF-α level was higher with drugs than LPS (Figure 4). Although these changes were not statistically significant (Figure 4), but it might mention the role of increased inflammatory cell aggregation in liver tissue when LPS-treated animals received antimalarial drugs. Hence, we might be able to propose that drug-induced tissue injury might recruit more inflammatory cells to the liver, which leads to more inflammation and cytokine production.

We found that methyl palmitate significantly decreased the level of ALT, LDH, and AST levels. This might indicate the role of inflammatory cells such as macrophages, which are inhibited by methyl palmitate, in antimalarial drugs-induced liver injury. On the other hand, the pathogenic role of other inflammatory cells such as neutrophils in the drug-inflammation interaction model is also mentioned in previous investigations.[23,50] Hence, neutrophils and other immune cells might also be responsible for the liver injury induced by AQ and CQ. 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). Applying broader acting inflammatory cell inhibitors and antibodies might shed light on the role of the immune system in antimalarial drugs hepatotoxicity. Furthermore, using inbreed animals are better choices to judge the role of immune system-mediated mechanisms of liver injury. On the other side, we might be able to suggest that immunosuppressant drugs might have a role in the treatment of antimalarial drugs-induced hepatotoxicity.

Serum bilirubin is usually used as a biomarker of biliary tree injury.[35,51] As bilirubin level didn't change significantly in the current investigation (Figure 3), we might be able to declare that the nature of antimalarial drugs-induced hepatotoxicity in the drug-inflammation interaction model is predominantly of the hepatocellular type rather than cholestatic.[35,51]

The role of peroxidase enzymes in adverse drug reactions has been reviewed previously.[52] MPO is a peroxidase enzyme present in neutrophils, monocytes, and tissue macrophages (Figure 6).[52] The MPO activity assessment in the current investigation indicated accumulation of inflammatory cells in the liver tissue (Table 1, Figure 5). Previous investigations mentioned the role of MPO in drugs metabolism and its relevance to hepatic injury.[53-55] Interestingly, it has been found that amodiaquine-induced cytotoxicity was hastened in an *in vitro* model of isolated rat hepatocytes in the presence of peroxidase enzyme.[17] Hence, reactive metabolites produced in the inflamed liver might implicate in antimalarial drugs-induced liver injury. The role of inflammatory cells and myeloperoxidase enzyme in amodiaquine and chloroquine metabolism in liver and its relevance to hepatotoxicity in inflammation interaction model need further experiments to be precisely cleared (Figure 6).

It has been found that the liver is inflamed in malaria-infected patients.[25-27] Tissue inflammation is an inseparable part of malaria disease.[56] On the other hand, inflammatory stress might sensitize the liver to xenobiotics-induced injury.[57] Hence, we might be able to suggest that inflammatory stress occurring during antimalarial drug therapy might participate in antimalarial drugs-induced liver injury in patients and enhance the sensitivity to antimalarial drugs.

Investigating the effect of inflammation on antimalarial drugs hepatotoxicity in a broader time frame will enhance our understanding of the mechanism of toxicity and the pathological pattern of liver injury induced by

these drugs. Moreover, understanding the mechanisms by which amodiaquine and chloroquine interact with inflammatory stress (Figure 6), could result in predicting drugs-induced liver injury and developing new therapeutic options.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no declaration of interest.

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