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Metabolites and JAK/STAT pathway were involved in the liver and spleen damage in male Wistar rats fed with mequindox

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ABSTRACT

Mequindox (MEQ) is a novel synthetic quinoxaline 1,4-dioxides antibacterial agent and growth promoter in animal husbandry. This study was to investigate whether reactive oxygen species (ROS), the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway, suppressors of cytokine signaling (SOCS) and inflammatory cytokines were involved in toxicities of MEQ. Our data demonstrated that high dose of MEQ (275 mg/kg) apparently led to tissue impairment combined with imbalance of redox in liver. In liver and spleen samples, hydroxylation metabolites and desoxymequindox were detected, directly confirming the potential link of N → O group reduction metabolism with its organ toxicity. Moreover, up-regulation of JAK/STAT, SOCS family, tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) were also observed in the high-dose group. Meanwhile, significant changes of oxidative stress indices in liver were observed in the high-dose group. As for NADPH subunit, the mRNA levels of many subunits were significantly up-regulated at low doses but down-regulated in a dose-dependent manner in liver and spleen, suggesting an involvement of NADPH in MEQ metabolism and ROS generation. In conclusion, we reported the dose-dependent long-term toxicity as well as the discussion of the potential mechanism and pathways of MEQ, which raised further awareness of its toxicity following with the dose change.

1. Introduction

Since 1970s, quinoxaline 1,4-dioxide derivatives (QdNOs) are one of the useful classes of synthetic agents with wide range of biological activities like growth promoter, antibacterial, anti-candida, antitubercular, anticancer and antiprotozoal properties (Vicente et al., 2009). Owing to their worthwhile effects in animal husbandry, these compounds have been added to cattle, swine, and poultry feeds to improve the growth at subtherapeutic levels. Our previous studies *in vitro* have demonstrated that the N → O group reduction was found to be the main metabolic pathway of QdNOs metabolism (Liu et al., 2008, 2009, 2010a,b) and the reduction of QdNOs may lead to the formation of reactive oxygen species (ROS) and cell apoptosis (Huang et al., 2010).

Mequindox (MEQ), a new synthetic QdNOs antibacterial agent, was found to cause liver and adrenal toxicity by acute and subchronic oral administration at high doses (Huang et al., 2009; Ihsan et al., 2010). *In vitro* research revealed that more than 10 metabolites of MEQ were characterized and observed in rat, chicken and pig liver microsomes (Liu et al., 2010a). In addition, NADPH oxidase was considered as a major source of ROS generation in both physiologic and pathophysiologic situations (Abid et al., 2007; Cave et al., 2006; Ying et al., 2009; Teufelhofer et al., 2003). From the information obtained above, we may hypothesize that the metabolism of MEQ could also be happened *in vivo* through deoxidize reaction with the help of NADPH enzyme, finally leading to the cell and tissue damage. But up till now, there is no attempt to testify if a direct linkage of the metabolism and toxic mechanism of MEQ existed *in vivo*.

Sub-chronic study in our lab has shown that MEQ can result in cellular swelling, centrilobular liver cell necrosis and disorganized hepatic cord pattern in liver in Wistar rats (Ihsan et al., 2010). *In vitro* study revealed that the prooxidant properties and

Abbreviations: CIS, cytokine-induced SH2 protein; EICs, extracted ion chromatograms; IL-6, interleukin 6; JAK, Janus kinase; LC/MS–ITTOF, high-performance liquid chromatography combined with hybrid ion trap/time-of-flight; MDA, malondialdehyde; MEQ, mequindox; ROS, reactive oxygen species; SOCS, suppressors of cytokine signaling; SOD, superoxide dismutase; RT-PCR, real-time reverse transcriptional polymerase chain reaction; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor alpha.

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imbalance in the redox metabolism of QdNOs would lead to dose and time-dependent cell damage via the mitochondria-dependent pathway (Huang et al., 2010). However, the mechanism of MEQ results in liver damage *in vivo* still remains unclear.

The Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway was assumed to be involved in the ROS mediated tissue dysfunction. It was shown as an important mediator of ROS-induced apoptosis in Jurkat T cells and mouse splenocytes (Oh et al., 2009). In addition, suppressors of cytokine signaling (SOCS) may act as part of a negative feedback loop to attenuate signal transduction from cytokines that act through JAK/STAT signaling pathway. Prolonged activation of STAT1 and STAT3 induced by interleukin-6 (IL-6) were found in SOCS3-deficient cells (Lang et al., 2003; Croker et al., 2003). The SOCS family was also involved in liver damage (Naka et al., 1998; Starr et al., 1998) and negatively regulates IL-6 signaling *in vivo* (Croker et al., 2003). Moreover, inflammatory factors such as tumor necrosis factor (TNF-α) and IL-6 may play important roles in hepatic steatosis (Sánchez-Garrido et al., 2009; Garcia-Ruiz et al., 2003; Naugler et al., 2007).

Based on the above studies, we may hypothesize that ROS generated by MEQ metabolism, JAK/STAT, SOCS family and inflammatory factors may be involved in MEQ-induced oxidative stress and hazard effects on organ damage at high dose. Since the liver has been documented to be the most common toxicological target of all toxicants and the spleen is the most important lymphoid organ, the present study was to evaluate the hepatic and lienal toxicity in male Wistar rats fed with MEQ for 180 days. In order to find direct evidence for the linkage of MEQ metabolism and its toxicity, MEQ and its metabolites in liver and spleen samples were detected by using high-performance liquid chromatography combined with ion trap/time-of-flight mass spectrometry (LC/MS–ITTOF). Five NADPH enzymes in liver and spleen were monitored to further explore the relationship between drug metabolism and oxidative damage. Morphological changes and oxidative stress indices as well as IL-6 and TNF-α in liver and spleen were examined. Meanwhile, real-time reverse transcriptional polymerase chain reaction (RT-PCR) was used to estimate the dose–response interaction of gene expression. Components of JAK/STAT pathway and SOCS family were detected to further elucidate the crosstalk among JAK/STAT pathways, SOCS family and inflammatory factors involved in organ impairment originated by ROS.

2. Materials and methods

2.1. Materials

Mequindox ($C_{11}H_{10}N_2O_3$, mol. wt. 218.21, purity 98%) was purchased from Beijing Zhongnongfa Pharmaceutical Co. Ltd. (Huanggang, PR China). All chemicals were purchased from Sigma (St. Louis, USA) unless otherwise stated.

2.2. Animals and diets

Male Wistar rats (4 W of age, $n = 75$) were purchased from Center of Laboratory Animals of Hubei Province (Wuhan, PR China). Prior to initiation of dosing, all rats were quarantined for 13 days and evaluated for weight gain and any gross signs of disease or injury. After quarantine, rats were randomly divided into five groups according to their body weight using the randomized block model. The control group was administered with standard commercial feed without feed additives. Other four groups received the same diet supplemented with 25, 55, 110 and 275 mg/kg MEQ, respectively. Animal rooms were maintained at a temperature of 22 °C, a relative humidity of 50 ± 20%, and a 12-h light/dark cycle. All rats were housed five per group in shoebox cages. Beddings (hardwood shavings) were used after sterilization by autoclaving and changed on a weekly basis. Food and water were supplied *ad libitum* and the treatment period lasted for 180 days. The protocol for this study was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Huazhong Agricultural University).

2.3. Pathologic observation

The livers and spleens from different rats in each group were collected and fixed in 10% neutral-buffered formalin and slides prepared for histopathological examination. Histopathological examination was conducted by using routine paraffin embedding technique. Sections of 4 μm thickness stained with hematoxylin and eosin (H&E) were examined under an optical microscope (Olympus BX 41, Japan) for morphological alterations.

2.4. LC/MS–ITTOF analysis of MEQ and its metabolites in liver and spleen

Mequindox and its metabolites in samples were detected by using hybrid IT/TOF mass spectrometry coupled with a high-performance liquid chromatography system (Shimadzu Corp., Kyoto, Japan). The liquid chromatography system (Shimadzu) was equipped with a solvent delivery pump (LC-20AD), an autosampler (SIL-20AC), a DGU-20A_3 degasser, a photodiode array detector (SPD-M20A), a communication base module (CBM-20A) and a column oven (CTO-20AC).

Liver and spleen samples were frozen in liquid nitrogen, and stored at −70 °C until analysis. When analysis, 0.1 g of the unfrozen organ samples were homogenized with 5 mL of 40 °C distilled water at the highest speed for 3 min in a model omni mixer homogenizer 17106 (OMNI International, Waterbury, CT, USA). Then the homogenate was homogenized for 1 min after that 5 mL of 15% trichloroacetic acid was added. The homogenate was centrifuged at 10,000 × *g* for 15 min. After that the supernatant was collected, 10 mL acetonitrile was added and vortex-mixed for 5 min. After vigorous shaking, followed by centrifugation at 10,000 × *g* for 15 min, the supernatant was dried using N_2 at 40 °C-water baths. The residue was reconstituted in 5 mL distilled water. The total supernatant was applied to the methanol and water pre-washed HLB 3cc cartridge (Waters Corporation, Milford, MA, USA). The samples were then sequentially washed with 3.0 mL water and 5% methanol in water. The extracts of liver and spleen tissue samples were eluted into plastic tubes with 5 mL methanol. The eluate was evaporated to dryness under nitrogen at 40 °C and the samples were reconstituted in 500 μL solution [methanol:water (40:60, v/v)] and passed through 0.22 μm filter membrane for LC/MS–ITTOF. The extracted samples were detected and analyzed as described previously (Liu et al., 2010a).

2.5. Oxidative stress indices in liver

Malondialdehyde (MDA) level and total superoxide dismutase (SOD) activities in the liver were assayed by using commercial kits from Jiancheng-Bioeng Institute (Nanjing, China), respectively. Protein concentration was measured using Coomassie blue protein-binding assay using bovine serum albumin (BSA) as a standard.

2.6. Real-time reverse transcriptional polymerase chain reaction (real-time RT-PCR)

Five NADPH oxidase isoforms (p47phox, gp91phox, Nox-1, p22phox, and Nox-4), five components (JAK1, JAK2, JAK3, STAT1 and STAT3) of the JAK/STAT pathway and four members (SOCS-1, SOCS-2, SOCS-3, and CIS) of the SOCS gene family in liver and spleen were determined by real-time RT-PCR, respectively. This enabled the localization of drug-induced interference as upstream or downstream of the cascade in the intermediate step. Briefly, Total RNA was isolated from tissue using Trizol Reagent according to the manufacture's instructions. One microgram of RNA was reverse transcribed to cDNA with the use of ReverTra Ace™ First Strand cDNA Synthesis kit (Promega, USA). cDNA was amplified by quantitative real-time PCR (Bio-Rad, USA) using SYBR® Premix Ex Taq™ RT-PCR kit (Takara, Code QPK-201, Japan). Each 25-μL reaction mixture consisted of 12.5 μL SYBR® Premix Ex Taq™, 0.5 μL of each primer (10 μm), 2 μL of cDNA, and 9.5 μL Rnase Free dH_2O. Cycling conditions were as follows: step1, 30 s at 95 °C; step 2, 40 cycles at 95 °C for 5 s, 60 °C for 30 s; step 3, dissociation stage. Data from the reaction were collected and analyzed by the complementary computer software. Relative quantification of gene expression was calculated using $2^{-\Delta\Delta Ct}$ data analysis method as previously described (Huang et al., 2009) and normalized to β-actin in each sample.

Primers used in this study were provided in Table 1.

2.7. Statistical analyses

Statistical analysis was performed by comparing the treatment groups with the control group using SPSS 13.0 program for windows. All results were presented as mean ± SD. Group differences were analyzed using one-way analysis of variance (ANOVA) followed by LSD's post hoc tests. A P-value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of MEQ on pathological changes in liver and spleen

There were significant increases in the relative organ to body weight ratios of liver and spleen in male rats when compared with controls (Table 2). The result showed important evidence of drug-related toxicity in the high-dose group.

Table 1
Primers used for real-time RT-PCR analysis.

Primer	Sequence (5′–3′)	Amplified region
β-Actin	Fwd: GAGATTACTGCCCTGGCTCCTA Rev: ACTCATCGTACTCCTGCTTGCTG	1026–1175
p47phox	Fwd: TGGTGGGTGGTCAGGAAAGG Rev: AGGAATCGGACGCTGTTGC	787–998
gp91phox	Fwd: CGGGACTTCGGACCCATATTC Rev: ATTCCTGTGATGCCAGCCAAC	3387–3532
Nox-1	Fwd: AATCCCATCCAGTCTCCAAAC Rev: CCTGCGGATAAACTCCATAGC	463–591
p22phox	Fwd: ACCGTCTGCTTGGCCATTG Rev: TCAATGGGAGTCCACTGCTCAC	331–404
Nox-4	Fwd: ACTGCCTCCATCAAGCCAAGA Rev: CTTCCAAATGGGCCATCAATGTA	1155–1244
JAK1	Fwd: GTCTGTGTCCGAGATGTG Rev: TCCAAGGGGTAGTAAGTG	1963–2068
JAK2	Fwd: CCCTGGCTGTCTATAACTCC Rev: TCTGTACCTTATCCGCTTCC	590–696
JAK3	Fwd: GCGACTGTCATTCTCTTTTG Rev: TTGAGTGTCCACGTCCTCTA	117–288
STAT1	Fwd: TCCTTCTTCCTGAACCCCCC Rev: AAGCCCATGATGCACCCATC	1453–1742
STAT3	Fwd: AATCTCAACTTCAGACCCGC Rev: GATCCACGATCCTCTCCTCC	815–934
CIS	Fwd: ATCTTGTCCTTTGCTGGTTGTG Rev: CTAGCACCTTCGGTTCATTCTC	21–184
SOCS1	Fwd: CGCTCCCACTCTGATTACCG Rev: CGAAGCCATCTTCACGCTGA	176–366
SOCS2	Fwd: GCAGTTTGACAGCGTGGTT Rev: ATGCTGAGTCGGCAGAAGT	351–512
SOCS3	Fwd: CCAAGAACCTACGCATCCAG Rev: TCCGTCGGTGGTAAAGAAAA	269–425
TNF-α	Fwd: CTCCTCACCCACACCGTCAG Rev: GAGCAGGTCCCCCTTCTCCA	457–630
IL-6	Fwd: TTCCAGCCAGTTGCCTTCTT Rev: CTGTTGTGGGTGGTATCCTC	25–128

Primers were manufactured by Shanghai Generay Biotech Co. Ltd. (Shanghai, PR China). JAK, Janus kinase; STAT, signal transducers and activators of transcription; CIS, cytokine-induced SH2 protein; SOCS, suppressor of cytokine signaling; TNF-α, tumor necrosis factor alpha; IL-6, interleukin 6.

Table 2
Relative organ weights of liver and spleen of male Wistar rats induced by 180 days feed of mequindox (%, Mean ± SD).

Mequindox (mg/kg)	*N*	Relative organ weight of liver[a]	Relative organ weight of spleen[a]
0	10	2.52 ± 0.20	0.17 ± 0.02
25	10	2.47 ± 0.19	0.20 ± 0.04
55	10	2.63 ± 0.34	0.20 ± 0.03
110	10	2.70 ± 0.36	0.20 ± 0.02
275	10	2.90 ± 0.16*	0.22 ± 0.03*

* Significantly different from control group at $p < 0.05$.
[a] *Note*: Relative organ weights were expressed as percentage of body weights and data presented as mean ± SD of 10 male Wistar rats.

In order to see whether MEQ diet cause any direct pathological alterations in rats, H & E was used to stain the liver and spleen tissues of rats as described above. As shown in Fig. 1, marked morphological changes were observed at high dose (275 mg/kg) when compared with control. Bile duct hyperplasia in liver and lymphocyte hyperplasia in spleen were observed at high dose (275 mg/kg).

3.2. Metabolites of MEQ in liver and spleen

The results showed that no MEQ was found in both liver and spleen samples and the metabolites of MEQ were different in liver and spleen. Five metabolites (M3, M6, M7, M11 and M14) and one metabolite (M11) were observed in rat liver and spleen, respectively. The metabolites were shown in Fig. 2 and the metabolic pathways of mequindox in rat were presumed. The main metabolite (2-isoethanol 4-desoxymequindox, M11) of MEQ was not detected in liver and spleen samples at 25 mg/kg group. However, M11 in both liver and spleen samples were increased following the dose increasing from 55 to 275 mg/kg (Fig. 2). The M11 amount in liver samples was almost equivalent to that in spleen at 275 mg/kg group. 2-Isoethanol bisdesoxymequindox (M14) or 3-hydroxymethyl 1-desoxymequindox (M3) were detected in liver samples at low doses. Bisdesoxymequindox (M6) and M14 reached peak in liver samples at 275 mg/kg group. However, M14 and M6

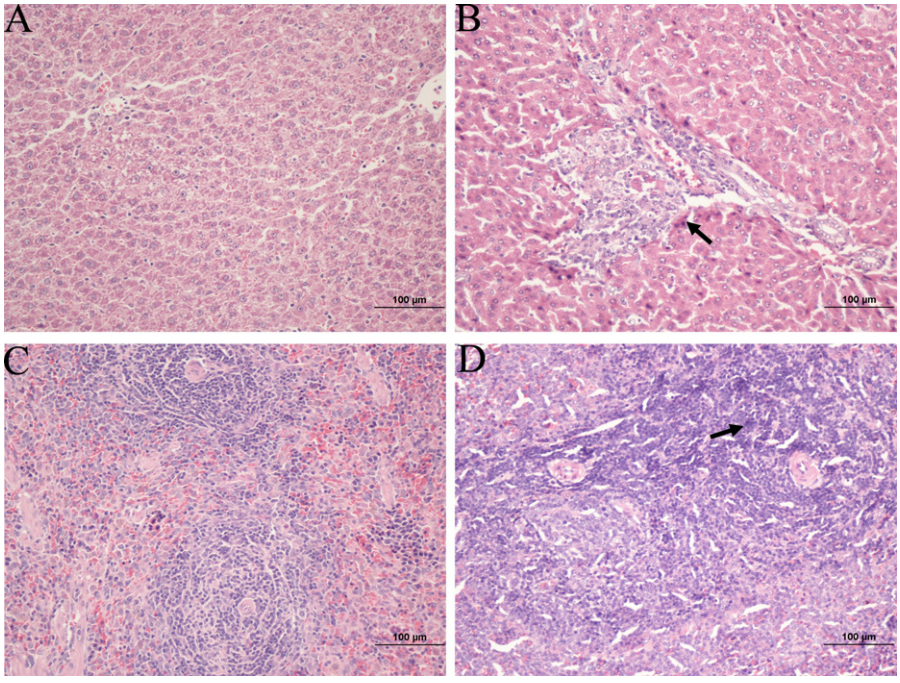

Fig. 1. Selected microphotographs of liver and spleen (200×). The different treatments of animals were shown: (A) Control liver. (B) Liver of 275 mg/kg MEQ, bile duct hyperplasia was marked with arrow. (C) Control spleen. (D) Spleen of 275 mg/kg mequindox, lymphocyte hyperplasia was marked with arrow.

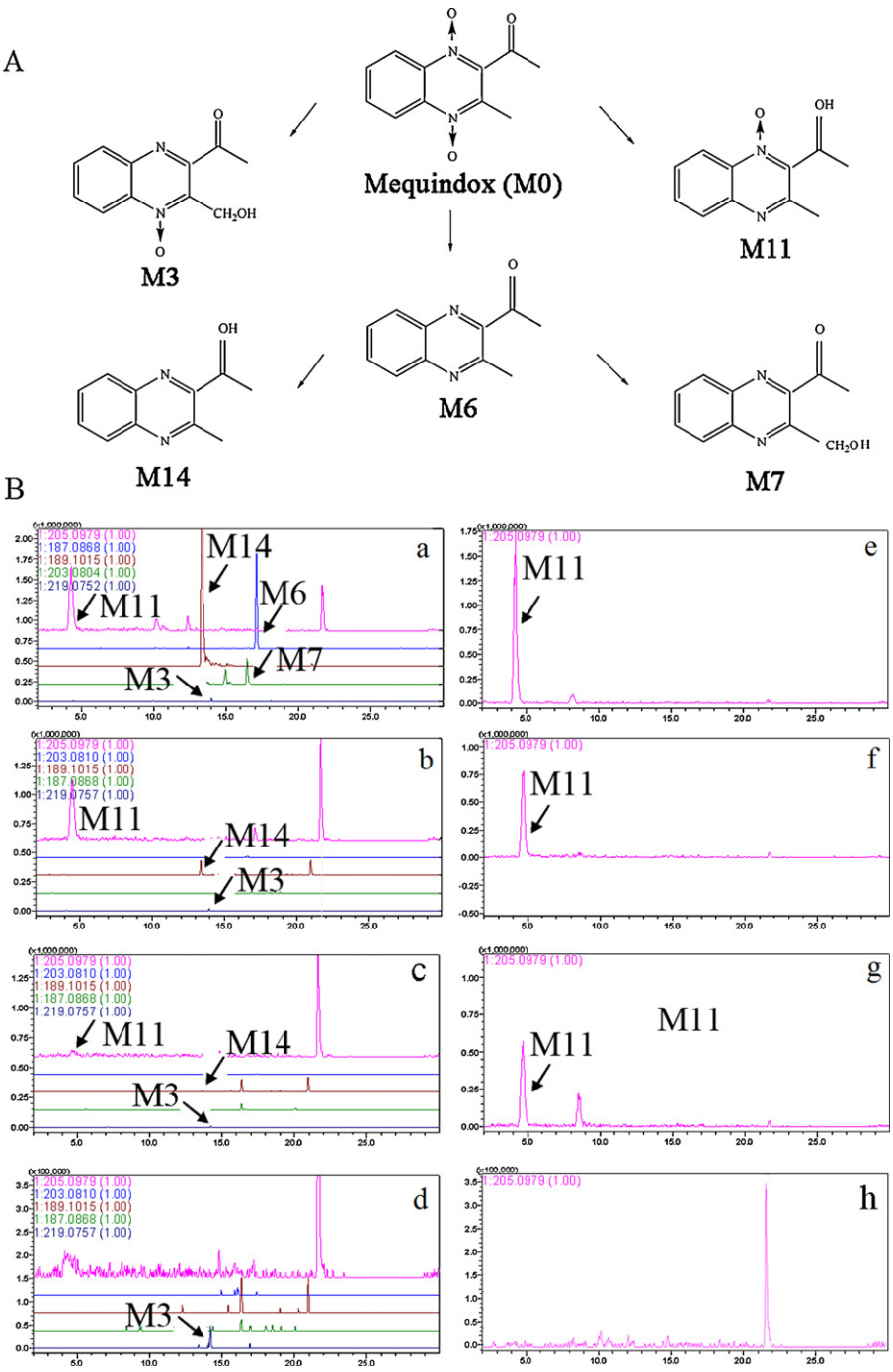

Fig. 2. The metabolites of mequindox in rat liver and spleen. (A) The chemical structure of mequindox and its metabolites; (B) The accurate extracted ion chromatograms (EICs) of mequindox metabolites formed in rat liver and spleen samples were shown: (a) Liver of 275 mg/kg mequindox. (b) Liver of 110 mg/kg mequindox. (c) Liver of 55 mg/kg mequindox. (d) Liver of 25 mg/kg mequindox. (e) Spleen of 275 mg/kg mequindox. (f) Spleen of 110 mg/kg mequindox. (g) Spleen of 55 mg/kg mequindox. (h) Spleen of 25 mg/kg mequindox.

Table 3
The retention times and fragment ions of MEQ's metabolites in liver and spleen samples.

Metabolite	RT (min)	$[M+H]^+$ (m/z)	Major fragment ions	Identification
M3	14.0	219	202, 201, 174, 159, 143	3-Hydroxymethyl 1-desoxymequindox
M6	17.0	187	159, 118	Bisdesoxymequindox
M7	16.5	203	186, 175, 158, 143	3-Hydroxymethyl bisdesoxymequindox
M11	4.2	205	188, 146	2-Isoethanol 4-desoxymequindox
M14	13.5	189	171	2-Isoethanol bisdesoxymequindox

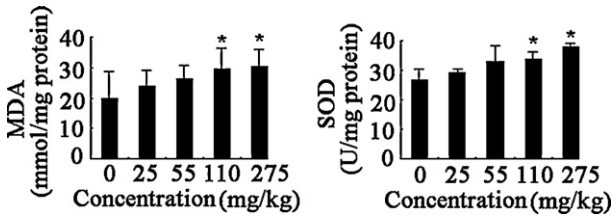

Fig. 3. Effects of mequindox on MDA levels (A) and total SOD activities (B) in rat livers. The results are presented as mean ± SD ($n = 6$). MDA, malondialdehyde. SOD, superoxide dismutase. Significant difference was indicated by $^{*}P < 0.05$, with respect to control.

were not found in spleen samples at 275 mg/kg group. M3 was only found in liver samples. The retention times and fragment ions of MEQ's metabolites in liver and spleen samples were provided in Table 3.

3.3. Effects of MEQ on MDA levels and total SOD activities in liver

Oxidative stress indices including total SOD activity as well as MDA, a terminal product of lipid peroxidation were examined in liver. As shown in Fig. 3, MDA levels in liver increased along with the increasing dose of MEQ diet. Moreover, total SOD activity was up-regulated in MEQ treatment groups, which indicated an imbalance of redox in liver.

3.4. Influence of MEQ on mRNA levels of NADPH oxidase in liver and spleen

The role of NADPH oxidase in MEQ-mediated oxidative stress was investigated in liver and spleen of rats. The gene expressions of its subunits were analyzed using real-time RT-PCR. The mRNA levels of NOX1 in liver in 25 and 55 mg/kg groups were significantly up-regulated. There were significant increases of the mRNA levels of NOX2 in liver in 25 mg/kg group and in spleen in 25, 55, 110 and 275 mg/kg groups. Significant increases of the mRNA levels of NOX4 in spleen in 25 and 55 mg/kg groups were noted. The mRNA levels of p22phox in spleen in 25, 55 and 110 mg/kg groups were significantly up-regulated. Significant increases of the mRNA levels of p47phox in liver in 25 mg/kg group and in spleen in 25, 55, 110 and 275 mg/kg groups were noted (Fig. 4). The mRNA levels of most units were significantly decreased as the MEQ dose level increased in liver and spleen (Fig. 4).

3.5. Influence of MEQ on mRNA levels of JAK/STAT and SOCS pathway components in liver and spleen

Real-time RT-PCR was used to check the mRNA levels of five components of JAK/STAT pathway, i.e. JAK1, JAK2, JAK3, STAT1, and STAT3, in liver and spleen, respectively. As shown in Fig. 5, both liver and spleen had an increase of mRNA levels of the five components of JAK/STAT pathway induced by the increasing dose of MEQ. Interestingly, the mRNA levels of JAK1 at low-dose groups were up-regulated not as much as the highest dose of MEQ in both liver and spleen. This phenomenon was also observed on JAK3 in spleen and on STAT3 in both liver and spleen. Except for JAK3 in spleen, the influence of MEQ on other components of JAK/STAT pathway in spleen were much weaker than that in liver.

The mRNA levels of SOCS1 and SOCS3 in liver were significantly down-regulated at low dose of MEQ, while they increased significantly at high dose (275 mg/kg). High dose of MEQ (275 mg/kg) resulted in an up-regulation of SOCS3 to more than fourfolds. The mRNA levels of SOCS2 in liver were up-regulated in a dose-dependent manner and it was more than threefolds at the high dose of 275 mg/kg.

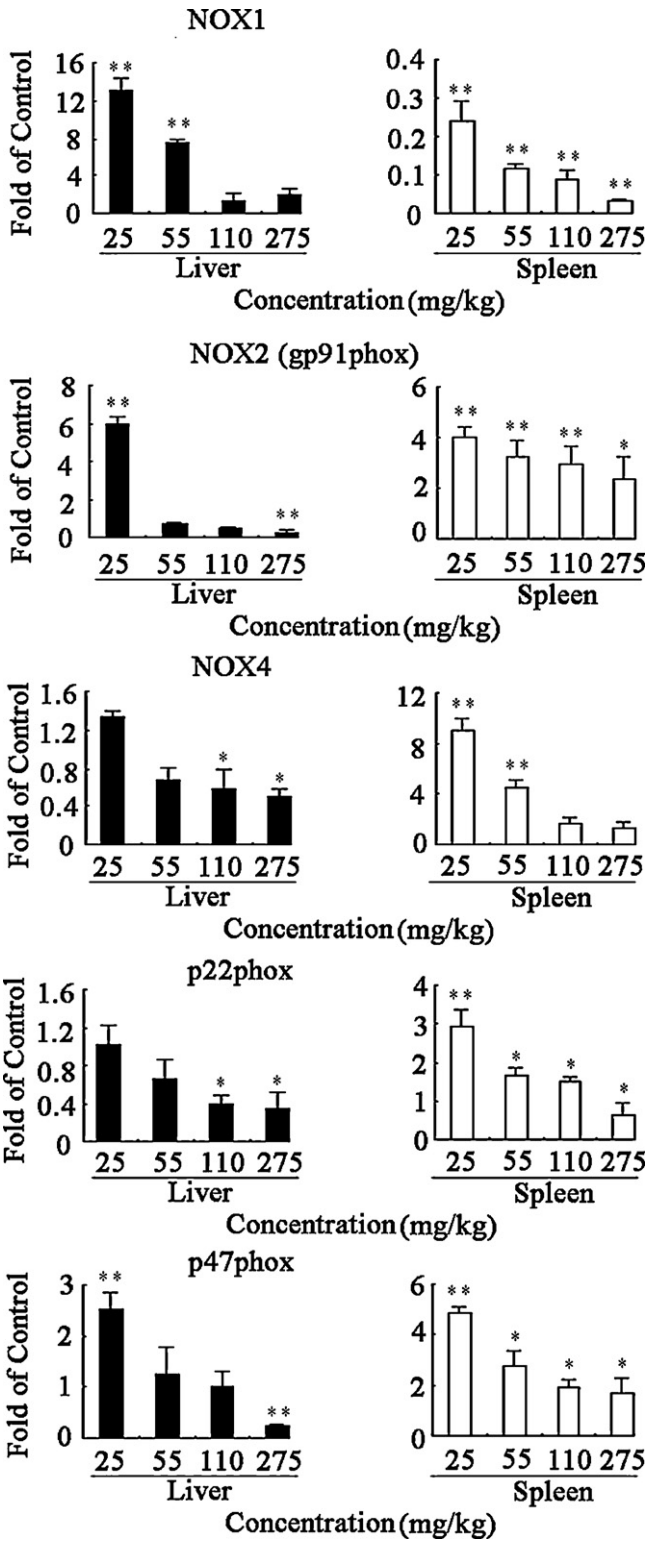

Fig. 4. Effects of mequindox on mRNA levels of NADPH oxidase in liver and spleen of rats. The results are presented as mean ± SD ($n = 5$). Significant difference was indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, with respect to control.

Similarly, the up-regulated trends were observed on SOCS1, SOCS2 and SOCS3 in a dose-dependent manner in spleen. However, opposite to the decrease in liver at 25 mg/kg dose of MEQ, the mRNA levels of SOCS1, SOCS2 and SOCS3 in spleen were all a little higher than the control group. These data indicated that SOCS may play a different role in different organs.

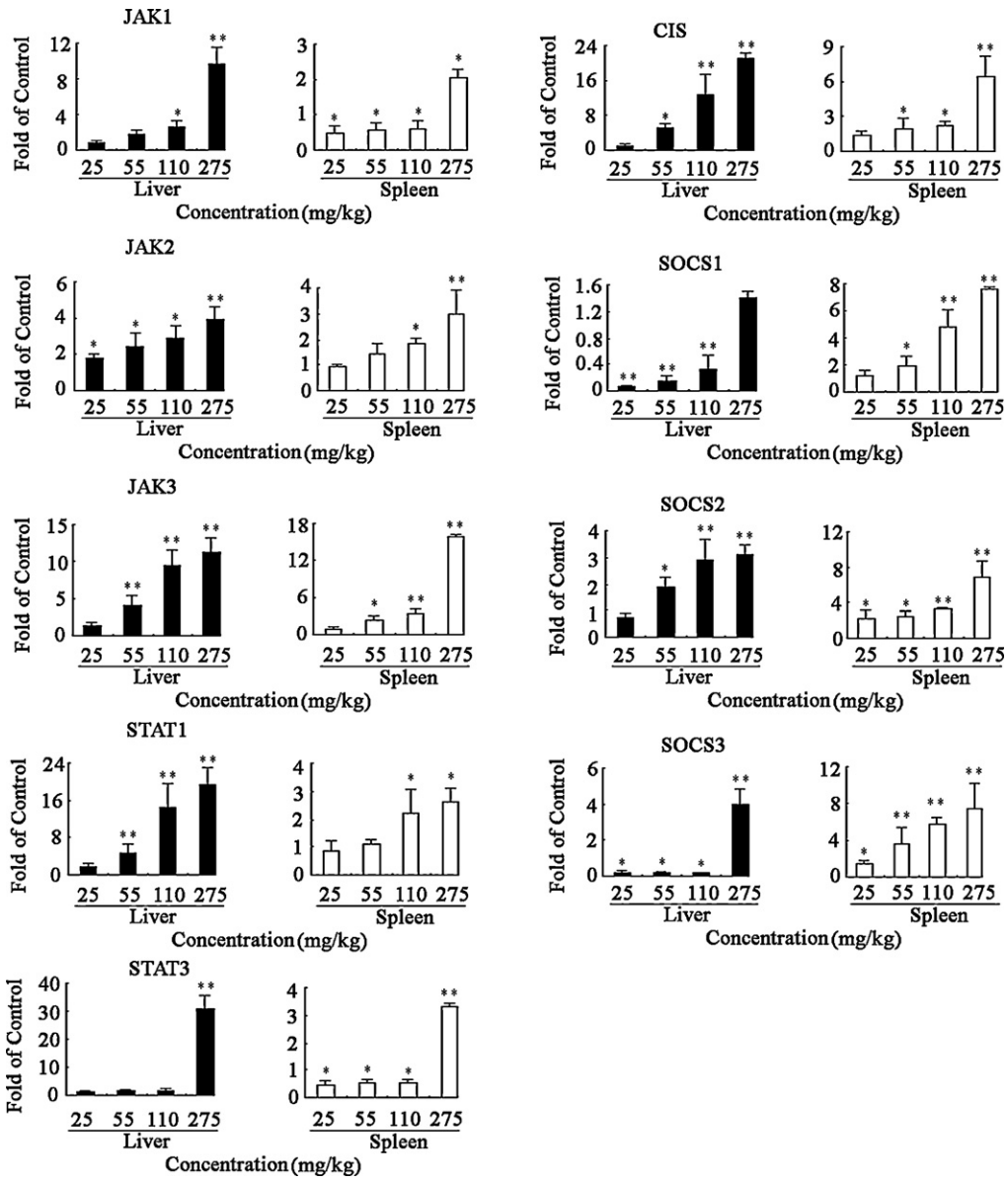

Fig. 5. Effects of mequindox on mRNA levels of JAK/STAT and SOCS components in liver and spleen of rats. The results are presented as mean ± SD ($n = 5$). JAK, Janus kinase. STAT, signal transducer and activator of transcription. SOCS, suppressors of cytokine signaling. CIS, cytokine-induced SH2 protein. Significant difference was indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, with respect to control.

3.6. Influence of MEQ on mRNA levels of TNF-α and IL-6 in liver and spleen

As shown in Fig. 6, the mRNA levels of TNF-α and IL-6 were remarkably up-regulated in a dose dependent manner both in liver and spleen. The significant decreases of TNF-α in liver were observed at the doses of 25 and 55 mg/kg, while a noticeable increase was noted at the 275 mg/kg group when compared with control.

4. Discussion

The main findings in this report are: (1) High dose of MEQ in the diet for 180 days led to liver and spleen impairment. (2) The N → O group reduction was the main metabolic pathway of MEQ metabolism *in vivo*, directly confirming the potential linkage of drug metabolism with its organ toxicity. (3) The mRNA levels of five components of JAK/STAT pathway as well as IL-6, TNF-α and SOCS family were up-regulated in liver and spleen, indicated they were involved in the dose-dependent long-term toxicity of MEQ. (4). Different doses of MEQ showed complicated and contradictory effect on NADPH oxidase in liver and spleen, further indicating an involvement in the metabolism of MEQ and its long-term toxicity in target organs.

Some *N*-oxide compounds are important as pharmacological or toxicological agents, such as chemotherapeutics, antibiotics, psychotropic drugs and carcinogens. It was presumed that their activities are dependent on the presence of their *N*-oxide groups (Scheutwinkel-Reich and vd Hude, 1984; Nunoshiba and Nishioka, 1989; Novave et al., 1986). The metabolic studies of quinoxalines in liver microsomes of different species showed that the N → O group reduction was the same metabolic pathway of quinoxalines (Liu et al., 2008, 2009, 2010a,b). The quinoxaline, a completely reduced derivative of quindoxin (quinoxaline-di-*N*-oxide), was not mutagenic, whereas the partially reduced quinoxaline-*N*-oxide exhibited a lower mutagenic activity than quindoxin (Beutin et al., 1981). Quinoxalie-2-carboxylic acid (QCA), a completely reduced derivative of carbadox, did not cause any significant production

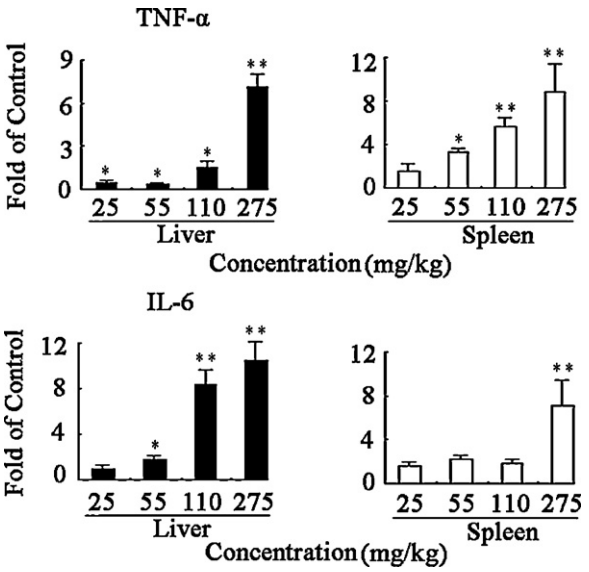

Fig. 6. Effects of mequindox on mRNA levels of TNF-α and IL-6 in liver and spleen of rats. The results are presented as mean ± SD ($n = 5$). TNF-α, tumor necrosis factor alpha. IL-6, interleukin 6. Significant difference was indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, with respect to control.

of ROS and showed relatively lower toxicity than its parents (Huang et al., 2010). Therefore, three kinds of bisdesoxymequindox (M6, M7 and M14) were thought to show lower toxicity than other two kinds of desoxymequindox (M3 and M11). Based on the accurate MS^2 spectra, the structures of metabolites and their fragment ions could be reliably characterized. However, because synthetic standards of metabolites are not available, we could not get the quantitative data of metabolites and had to rationalize the dose-relationship from their relative peak heights observed in the LC/MS–ITTOF. Because of relatively higher peak height of M11 than M3 at 110 and 275 mg/kg doses, M11 was thought as one of the main toxicological metabolites and the damage was resulted from the progress during the N → O group reduction. These data firstly proved a direct metabolism of MEQ in animal tissue. However, the results were much different from that obtained *in vitro* (Liu et al., 2010a). *In vitro* study, 1-desoxymequindox (M2) and M6 were the main metabolites and the relative percentage of M11 was very low when MEQ was incubated with rat liver microsomes. In the present study, M11, M6 and M14 were the main metabolites in rat liver in the high dose group. The results showed that the metabolic pathway of MEQ *in vivo* was much different with those ones *in vitro* especially when rats were administered a toxic dose. It was notable that the concentrations of M11 in both liver and spleen samples were similar in 275 mg/kg group. The potential mechanism may be that much more desoxymequindox was produced in the liver, such as M11, and resulted in the liver damage. The excessive accumulation of M11 was carried into other organs, such as spleen. The results directly testified the relationship between the metabolism of MEQ and its potential toxicity *in vivo*.

The *in vitro* study has found that NADPH enzyme was necessary in the N → O group reduction of QdNOs and protonated MEQ lost two $OH^{\bullet}$ radicals in a concerted action to H_2O_2 (Liu et al., 2009). It was indicated that ROS was involved in pathological processes induced by QdNOs (Chowdhury et al., 2004; Azqueta et al., 2007; Priyadarsini et al., 1996; Ganley et al., 2001). Derived from NADPH oxidase subunits, ROS played important roles in cell apoptosis and oxidative stress (Abid et al., 2000; Arnold et al., 2001; Badham and Winn, 2010; Huang et al., 2009, 2010). However, the role of NADPH *in vivo* still remains unclear. In the present study, some NADPH oxidase subunits were significantly up-regulated at low doses but down-regulated at high dose (275 mg/kg) in liver and spleen. The result showed that NADPH oxidase was involved in the reduction of MEQ *in vivo*. They might be exhausted at high dose of MEQ or their activities were negatively mediated by the generation of ROS (Huang et al., 2010), which need further investigation.

Activation of NADPH oxidase and ROS can activate JAK/STAT signaling pathway (Byfield et al., 2009; Madamanchi et al., 2001). Once JAKs are activated, they can activate other signaling molecules, including the STAT family of nuclear transcription factors (Darnell et al., 1994; Schindler and Darnell, 1995), then result in liver dam-

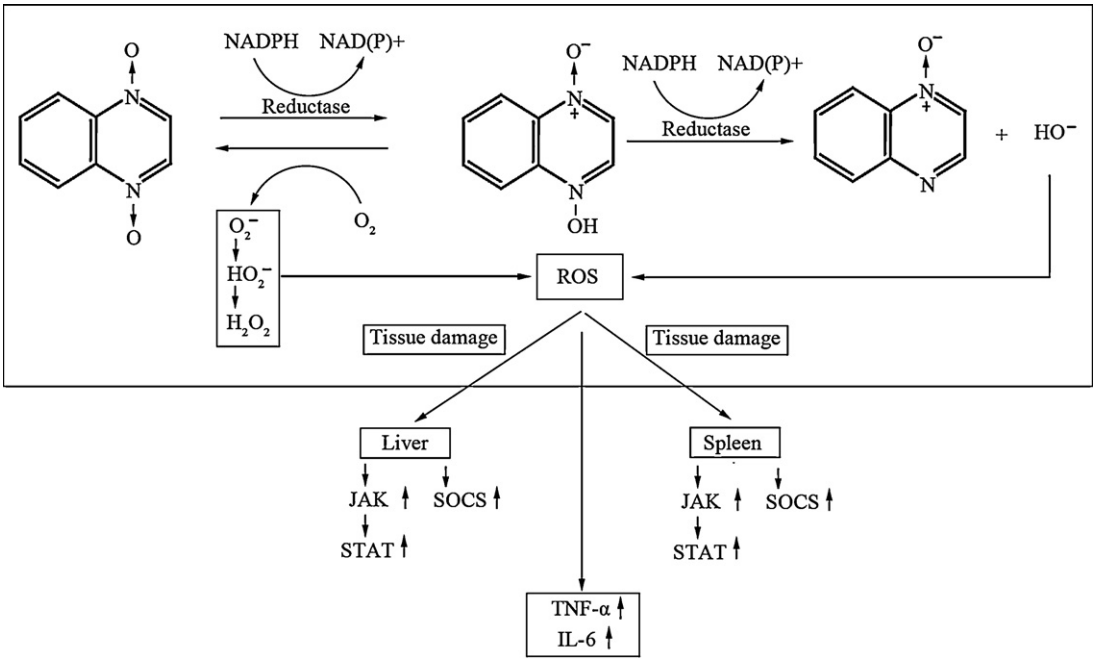

Fig. 7. Metabolites and JAK/STAT pathway were proposed to be involved in the liver and spleen damage in male Wistar rats fed with mequindox. JAK, Janus kinase. STAT, signal transducer and activator of transcription. SOCS, suppressors of cytokine signaling. TNF-α, tumor necrosis factor alpha. IL-6, interleukin 6.

age (Freitas et al., 2010). On the other hand, SOCS family negatively regulates JAK/STAT pathway (Minamoto et al., 1997). The present results showed that mRNA levels of JAK/STAT components as well as SOCS genes were up-regulated in a dose-dependent manner in liver and spleen, firstly indicating that JAK/STAT and SOCS signaling pathway were possibly involved in the MEQ-induced damage of liver and spleen (to see in Fig. 7).

The current study observed remarkable increase of IL-6 and TNF-α in both liver and spleen induced by high dose of MEQ, indicating that there was a linkage between the up-regulation of the proinflammatory cytokines in spleen and liver damage. In addition, significantly increased level of IL-6 (Sawczenko et al., 2005) and TNF-α (Ruuls and Sedgwick, 1999) usually caused stunted growth. Furthermore, JAK2 was recruited and STATs could be activated and translocated to the nucleus and up-regulated the expression of genes involved the growth promotion (Darnell et al., 1994; Schindler and Darnell, 1995). The present results may further elucidate our results that MEQ could increase the body weight at low dose but significantly reduce the body weight at chronic high-dose in Wistar rats.

In conclusion, the current study identified metabolites of mequindox *in vivo*, which indicated that high doses of MEQ lead to oxidative damage and dysfunctions in liver and spleen. It seems that NADPH oxidase, as well as JAK/STAT signaling pathway, SOCS family and inflammatory factors were cross-linked with MEQ-induced-redox imbalance, liver and spleen damage.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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