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Hepatotoxicity in Rats Treated with Dimethylformamide or Toluene or Both

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The effects of toluene in dimethylformamide (DMF)-induced hepatotoxicity were investigated with respect to the induction of cytochrome P-450 (CYP) and the activities of related enzymes. The rats were treated intraperitoneally with the organic solvents in olive oil (Single treatment groups: 450 [D1], 900 [D2], 1,800 [D3] mg DMF, and 346 mg toluene [T] per kg of body weight; Combined treatment groups: D1+T, D2+T, and D3+T) once a day for three days, while the control group received just the olive oil. Each group consisted of 4 rats. The activities of the xenobiotic metabolic enzymes and the hepatic morphology were assessed. The immunoblots indicated that the expression of CYP2E1 was considerably enhanced depending on the dosage of DMF and the CYP2E1 blot densities were significantly increased after treatment with both DMF and toluene, compared to treatment with DMF alone. The activities of glutathione-S-transferase and glutathione peroxidase were either decreased or remained unaltered after treatment with DMF and toluene, whereas the lipid peroxide levels were increased with increasing dosage of DMF and toluene. The liver tissue in the D3 group (1,800 mg/kg of DMF) showed signs of microvacuolation in the central vein region and a large necrotic zone around the central vein, in rats treated with both DMF (1,800 mg/kg) and toluene (D3T). These results suggest that the expression of CYP2E1 is induced by DMF and enhanced by toluene. These changes may have facilitated the accelerated formation of N-methylformamide (NMF) from toluene, and the generated NMF may directly induce liver damage.

***Key words*:** Dimethylformamide, Toluene, Cytochrome P-4502E1, Enzyme, Lipid peroxides

INTRODUCTION

Dimethylformamide (DMF, CAS 68-12-2) is a polar solvent widely used as an organic solvent in industry (e.g. for the production of acrylic resin, synthetic leather, films, synthetic textiles etc.) (1). Intake of DMF may be mainly from inhalation of its vapor and by physical contact with skin, and occupational exposure to DMF may cause nausea, abdominal pain, and alcohol intolerance (2). Especially, DMF has been shown to induce hepatotoxicity and liver dysfunction in animals and humans by many investigators, and it is known that the primary target organ of DMF is the liver in animals and humans (3-5). DMF is hydroxylated at its methyl moieties by the cytochrome P-450 (CYP) to produce N-methyl-N-hydroxymethylformamide (DMF-OH), the generated DMF-OH being biotransformed to N-methylformamide (NMF) by a non-enzymatic or enzymatic reaction, undergoing further reactions that result in xenobiotic enzymes (6,7). The hepatotoxicity by the DMF itself and/or its intermediates are hepatotoxic. In the liver activated intermediates such as NMF are more toxic than DMF itself (8,9). The activated intermediates have high polarity and reactivity. This hepatotoxicity is induced through covalent binding with macromolecules such as DNA, RNA, and proteins. But, actually, in most chemical factories, mixture rather than a single substance is open used. However most of the risk assessment study was focused on a single substance. Mixture can have different physical and chemical properties that different from the unique characteristics of each chemical. Therefore, its toxic effects on the human body can also be changed. It was reported that the metabolic ratio of DMF as a single exposure is differ from co-exposure with methyl ethyl ketone (MEK) and/or toluene (10). And

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simultaneous exposure of alcohol and DMF can cause synergetic effect to liver function abnormality (11). In our previous study, we found workers in synthetic leather and synthetic textile factories that were simultaneously exposed to DMF and toluene and/or DMF and MEK. However, the effect of simultaneous exposure to DMF and other chemicals was not well investigated (12).

Thus, we studied the effects of toluene on induced hepatotoxicity by DMF, focusing on the activities of CYP-dependent catalytic enzymes and oxidative stress.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), 1-chloro-2, 4-dinitrobenzene (CDNB), cumene hydroxide, cytochrome C, dimethylformamide, EDTA, ethoxyresorufin, ferrous ammonium sulfate, glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD), glutathione S-transferase (GST), glutathione reductase, malonaldehydebis (diethyl acetal), NADH, NADPH, p-nitrophenol, 7-pentoxyresorufin, sodium dodecyl sulfate (SDS), thiobarbituric acid, toluene, and xanthine oxidase (XOD) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Western immunoblotting kits employing enhanced chemiluminescence detection were purchased from TROPIX Inc. (Bedford, MA, USA). All other chemicals were obtained commercially and they were of analytical grade.

Animals. Male Sprague-Dawley rats (7 week old) were obtained from a specific pathogen-free colony from the Central Lab Animal In. (Seoul, South Korea), and were kept in cages for 1 week prior to treatment. These animals were kept under controlled conditions with respect to temperature (23 ± 2^{o}C), humidity ($50 \pm 5\%$), and 12 hrs light/dark cycle. They had free access to sterilized water and food (Mouse Pellets, Jeil Lab Chow, and Taejon, South Korea) *ad libitum*. The rats were treated intraperitoneally with organic solvents in olive oil [Single treated groups: 450 (D1), 900 (D2), 1,800 (D3) mg DMF, and 346 mg toluene (T) per kg of body weight; Combined treated groups: D1+T, D2+T, and D3+T] once a day for three days. Dose levels were determined on the authority of the short-term exposure limit concentration recommended by ACGIH (13). Each group consisted of 4 rats, but D3+T group was only one rat because the three animals died few hours after administration. The control group received olive oil. Before rats were obtained for research, the rat studies were approved by an Animal Ethnics Committee (IACUC) in order to ensure appropriate animal care.

Blood collection and preparation of liver microsomes. The rats were fasted for 24 hrs before death, anesthetized with pentobarbital and their blood was collected immediately from the heart. Blood was drawn into tubes containing potassium EDTA as an anticoagulant and it was used for the analyses of the liver function test. After blood collection, livers were quickly removed, placed in ice-cold 0.9% NaCl solution, homogenized in 0.25 M sucrose, and subjected to centrifugation at 12,000 ×g for 40 min. The microsomal fraction was obtained from the 12,000 ×g supernatant by centrifuging for 60 min at 105,000 ×g, resuspending in 0.25 M sucrose (14), and storing at -70^{o}C until use.

Liver function test. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) in the serum of the rats were determined by adapting the standard methods to diagnostic reagent kits using a COBAS MIRA PLUS (Roche Co., USA).

Enzyme assays. Protein concentration was determined by the Kim *et al*. (15) procedure using BSA as a standard. The total content of CYP in the liver microsomal fraction was measured by using the carbon monoxide-reduced difference spectrum according to Kim *et al.* (16). Ethoxyresorufin-O-deethylase (EROD) activity was determined by the method of Lowry *et al.* (17) with slight modification. The reaction mixture (2 ml) consisted of 0.1 M potassium phosphate buffer (pH 7.5) containing 2 mg/ml of BSA, 10 μM dicumarol, 5 mM G-6-P, 5 units/ml of G-6-PD, 5 μM NADPH, and 2.5 μM 7-ethoxyresorufin. The reaction was started by adding the substrate into the reaction tube and was performed at 37^{o}C for 20 min. The formation of resorufin was monitored fluorometrically at an excitation of a maximum of 550 nm and an emission maximum of 585 nm. The results were presented as nmoles of resorufin generated/min per mg protein. The activity of pentoxyresorufin-O-dealkylase (PROD) was determined by the method of Omura and Sato (18). All reaction components and assay procedures were the same as in the EROD assay except for the substrate, 2.0 μM of pentoxyresorufin. The results were also presented as nmoles of resorufin generated/min per mg protein. The activity of ρ-nitrophenol hydroxylase (ρNPH) was measured by spectrophotometric determination of 4-nitrocatechol formed from the hydroxylation of p-nitrophenol (19). The content of lipid peroxide (LPO) was spectrometrically measured as described by Lubet *et al.* (20). Glutathione S-transferase (GST) activity was determined by the method of Koop (21) described briefly as follows. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.5), 10 mM of glutathione, and 50 μl of cytosolic fraction with a final volume of 1.0 ml with 0.1 M potassium phosphate buffer (pH 6.5). The reaction was started by addition of 20 mM of CDNB and GST activity was determined spectrophotometrically at 340 nm by using an extinction coefficient of 9.6 $cm^{-1}mM^{-1}$. Glutathione peroxidase (GSHPx) activity was spectrophotometrically measured as described by Yagi (22).

Western immunoblot analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 8.5%) was performed according to Laemmli's (23) method using a Bio-Rad Protein II Cell apparatus. Western immunoblotting of CYP isozymes was carried out as described previously using monoclonal anti-mouse CYP1A1/2 (MAb 1-7-1), CYP2B1/2 (MAb 2-66-3), and CYP2E1 (MAb 1-98-1) antibodies (24). Liver microsomal proteins were separated by 8.5% SDS-PAGE and electrophoreticallytransferred to nitrocellulose membranes. The membranes were incubated with the primary antibodies (ascite, 500 μg/ml) over night, followed by incubation with biotinylated goat anti-mouse IgG (TROPIX Inc.) at a 1:5,000 dilution for 1 hr after washing. The membranes which had been subjected to immunological reactions were incubated in a chemiluminescence substrate solution containing 1:20 Nitro-Block for 5 min and the specific protein bands were visualized by exposing the membranes indirectly through a plastic cover to standard x-ray film.

Histopathological studies. Male control Sprague-Dawley rats and those treated with organic chemicals as described above were divided according to condition and killed 24 hrs after their last treatment and their livers were collected, rapidly fixed in 10% buffered formalin, processed, and embedded in paraffin. They were stained with hematoxylin and eosin and the effect of the chemicals on hepatic morphology were assessed by light microscopy.

Statistical analysis. All the data were analyzed using Version 12.0 of the SPSS statistics program (SPSS Inc.,

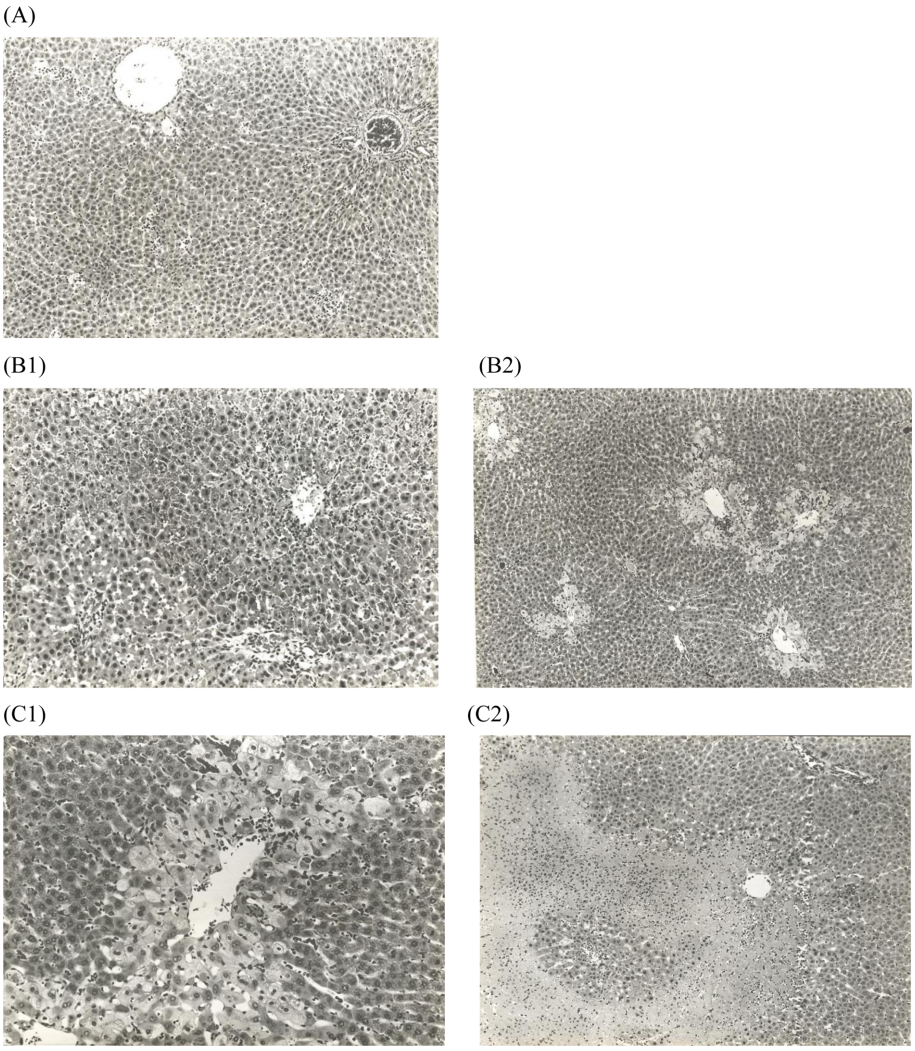

Fig. 1. Light microscopic image of H & E staining on liver section after administration as described in Materials and Methods. (A) Control, (B1) DMF administration (900 mg/kg), (B2) Both DMF (900 mg/kg) and toluene (436 mg/kg), (C1) DMF administration (1,800 mg/kg), (C2) Both DMF (1,800 mg/kg) and toluene (436 mg/kg), (C1) DMF administration (1,800 mg/kg): cytoplasmic vacuolization of hepatocytes and altered cell focus around the central vein and (C2) Both DMF (1,800 mg/kg) and toluene (436 mg/kg) administration: zonal necrosis around the central vein.

Chicago, USA). Data that were expressed as mean ± SD were analyzed using a one-way analysis of variance (ANOVA) with post hoc testing by the Scheffé test.

RESULTS

Pathological changes of liver. The morphological changes of the liver caused by DMF and both DMF and Tol treatment are shown in Fig. 1. The effects of hepatotoxic DMF and Tol did not provoke hepatotoxicity clearly in rats of the D1, D2, T, D1T, and D2T treated by DMF and Tol, whereas in the D3 group receiving 1,800 mg/kg of DMF, signs of vacuolization of hepatocytes and altered cell focus around the central vein region (Fig. 1C1) and serious zonal necrosis around the central vein in rats treated with both DMF (1,800 mg/kg) and Tol (D3T) could be observed (Fig. 1C2).

Activities of CYP-dependent catalytic enzymes and immunoblot assay. The effects of increasing doses of DMF and simultaneously treatment with Tol on total microsomal CYP content and the activity of CYP-dependent catalytic enzymes are presented in Table 1. By repeated treatment, the microsomal CYP contents in single DMF treated groups were significantly increased according to increasing dosage ($p < 0.05$), whereas it was decreased in groups treated with both DMF and Tol and was dependent on DMF dosage. EROD, PROD, and ρNPH are considered to be associated with the activity of CYP1A1 (EROD), CYP2B1/2 (PROD), and CYP2E1 (ρNPH), respectively (18,15,25). In this study, no significant changes in EROD and PROD activities were observed among the control, single treated with DMF, and treated with both DMF and Tol, but EROD activity between the D1 and D1T group was only statistically significant ($p < 0.05$). ρNPH activity associated with CYP2E1 increased steadily with the increase in the concentration of DMF and the increase was prominent in the group single treated with DMF ($p < 0.05$). Fig. 2 shows the results of immunoblot analyses which were carried out with microsomes from control rats and rats treated with DMF and both DMF and Tol using MAb specific to CYP2E1. The densities for CYP2E1 were considerably enhanced according to the dosages of DMF and the increase existed in the treated groups, with a larger increase for the rats treated with both DMF and Tol than the groups of rats which were treated with a single compound.

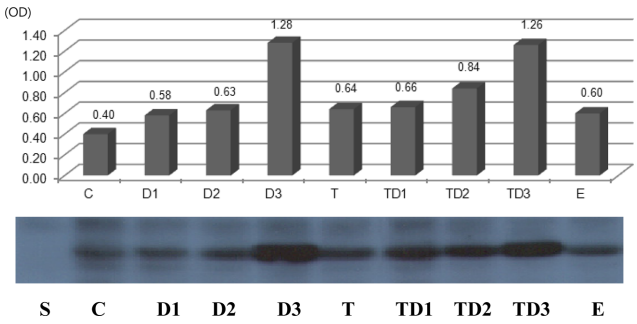

Fig. 2. Western immunoblot analysis for hepatic microsomes of rats treated with DMF and both DMF and toluene utilizing mouse monoclonal anti-rat CYP2E1 antibody. Liver microsomes (10 μg) were loaded for the groups: C, control; D1, DMF 450 mg/kg; D2, 900 mg/kg; D3, 1,800 mg/kg; T, toluene 436 mg/kg; TD1, T+D1; TD2, T+D2; TD3, T+D3; E, ethanol (0.5 μg loaded) and S, size marker.

Liver function and activity of glutathione-dependent enzymes. GST, GSHPx activities and content of LPO are presented Table 2. The activities of GST in treated groups were higher than that of the control group, except the D3T group. GSHPx activity was also not affected by the increasing dosages of DMF and Tol. The contents of LPO were not affected by DMF, Tol, and both ($p < 0.05$). The effects of DMF or both DMF and Tol on liver function are presented in Table 3. The activities of liver functions such as AST,

Table 1. CYP content and activities of CYP-dependent catalytic enzymes in liver microsomes from rats treated with various DMF doses

Groups (mg/kg)	CYP	EROD	PROD	ρNPH
Control	0.54 ± 0.06^{abdfg}	0.25 ± 0.05^{abcdefgh}	0.95 ± 0.20	715.6 ± 19.9^{abeg}
Tol (436)	0.57 ± 0.03^{abdfg}	0.23 ± 0.02^{abcdegh}	0.20 ± 0.03	646.3 ± 61.1^{aeg}
DMF1(450)	0.57 ± 0.03^{dfg}	0.20 ± 0.07^{abcdeg}	1.18 ± 0.38	$1250.4 \pm 163.8^{abcfgh}$
DMF1 +Tol	0.83 ± 0.11^{ce}	0.38 ± 0.06^{adfgh}	0.23 ± 0.04	1373.6 ± 190.7^{bcfgh}
DMF2(900)	0.86 ± 0.04^{e}	0.21 ± 0.04^{abcdeg}	1.13 ± 0.14	1762.2 ± 52.1^{bcfh}
DMF2 +Tol	0.69 ± 0.04^{abdfg}	0.27 ± 0.03^{abcdefgh}	0.25 ± 0.04	1046.5 ± 13.3^{abefgh}
DMF3(1,800)	1.23 ± 0.02^{h}	0.26 ± 0.06^{abcdefgh}	1.33 ± 0.27	2674.0 ± 250.9^{d}
DMF3 +Tol†	0.52	0.34	0.25	1381.3
F-values	47.00^{*}	8.31^{*}	4.35	46.80^{*}

$^{*}p < 0.05$. †only one rat. Data are presented as mean ± SD of four rats (n = 4). CYP content is expressed as nmol/mg of protein and catalytic enzyme activities as nmol/(min·mg of protein). Upper letters (a, b, c, d, e, f, g, h): The same letters are not significantly different (Scheffé test, $p < 0.05$).

Table 2. Effect of DMF on activities of glutathion S- transferase (GST), glutathion peroxidase (GSHPx) and the generated lipid peroxide (LPO) level and the effect of toluene in controlling changes induced by DMF

Groups (mg/kg)	GST	GSHPx	LPO
Control	1.26 ± 0.04^{aceg}	2.75 ± 0.03^{acd}	0.58 ± 0.07
Tol (436)	1.22 ± 0.02^{aeg}	1.98 ± 0.11^{befgh}	0.73 ± 0.09
DMF1(450)	1.52 ± 0.05^{bcdg}	2.23 ± 0.03^{bcefgh}	0.63 ± 0.03
DMF1 +Tol	1.76 ± 0.07^{df}	2.25 ± 0.02^{bcefgh}	0.75 ± 0.03
DMF2(900)	1.44 ± 0.04^{abcdg}	2.47 ± 0.10^{abcdfg}	0.73 ± 0.11
DMF2 +Tol	1.41 ± 0.05^{abcdeg}	2.12 ± 0.12^{bcefgh}	0.70 ± 0.14
DMF3(1,800)	1.59 ± 0.04^{bcdfg}	2.61 ± 0.16^{acd}	0.83 ± 0.18
DMF3 +Tol†	0.97	2.05	1.52
F-values	50.74*	21.51*	22.09

*$p < 0.05$. †only one rat. Data are presented as mean ± SD of four rats (n = 4). LPO content is expressed as nmol/mg of protein and GST and GSHPx activities as nmol/(min·mg of protein). Upper letters (a, b, c, d, e, f, g, h): The same letters are not significantly different ($p < 0.05$).

Table 3. Clinical data of liver function in control and treated groups

Groups (mg/kg)	AST (U/L)	ALT (U/L)	ALP (U/L)
Control	162.0 ± 29.8	51.7 ± 12.0	122.3 ± 21.5
Tol (436)	198.7 ± 19.9	42.0 ± 5.0	174.0 ± 42.6
DMF1(450)	178.0 ± 21.8	46.3 ± 14.1	136.0 ± 59.8
DMF1 +Tol	241.6 ± 57.0	80.7 ± 11.1	203.0 ± 67.1
DMF2(900)	195.0 ± 28.1	55.7 ± 23.0	175.7 ± 75.7
DMF2 +Tol	287.7 ± 28.4	129.3 ± 12.3	261.3 ± 52.3
DMF3(1,800)	320.7 ± 161.5	159.0 ± 84.2^{a}	135.7 ± 14.2
DMF3 +Tol†	483.0	244.0	275.0
F-values	2.31	5.36*	2.58

*$p < 0.05$. †only one rat. Data are presented as mean ± SD of four rats (n = 4). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase. [a]: Significantly different from the other groups ($p < 0.05$).

ALT, and ALP were increased steadily by the increased dosages of DMF, and with both DMF and Tol, the increases were more readily apparent with DMF alone, even though they were not statistically different ($p < 0.05$).

DISCUSSION

When living organisms are exposed to environmental xenobiotics, they are able to produces specific enzymes and metabolize and excrete the metabolites from their bodies via detoxification processes. The CYP are major enzymes for the metabolism of xenobiotics as well as for certain classes of endobiotics, including fatty acid, steroids, and prostaglandins (26-29). More than 100 forms of CYP have been identified and classified into over 20 different families and subfamilies, and it is known that they are involved in the metabolism of xenobiotics and endobiotics through over 60 different reactions that metabolize hundreds of thousands of different chemicals (30). Among than, CYP2E1 isozyme plays a key role in the bioactivation of a variety of low molecular weight protoxicants and procarcinogens (31), including many solvents such as benzene, trichloroethylene, acetone, DMF, and ethanol (32). Guengerich *et al*. (33) reported that CYP2E1 catalyzed the metabolic oxidation and toxification of N-methylformamide by an inhibition study of the enzyme, Nakajima (34) reported that CYP1A, CYP2B and CYP3A2 were involved in the metabolisms of acetaminophen, carbon tetrachloride and DMF. He also found that liver damage was induced remarkably in the group of animals treated with DMF after being retreated with acetone, with the suggestion that the CYP2E1 isozyme was involved in the metabolism of DMF (35).

We previously observed that the CYP2E1 isozyme was inducible by DMF using a monoclonal anti-mouse CYP2E1 antibody (5). Many studies of DMF have been carried out as a single compound even though most workers are exposed simultaneously to DMF as well as Tol and/or MEK. Studies of simultaneous exposure have not been clearly demonstrated (12,35). Thus, we examined the effect of Tol on DMF-induced hepatotoxicity, monitoring the induction of CYP and the other related enzymes in rat liver. (i) CYP content and activities of PROD and ρNPH were increased by increasing the dosage of DMF, but the combination of DMF and Tol decreased activity compared to single treatment with DMF. (ii) The densities of CYP2E1 were also considerably enhanced by the dosages of DMF and the blot densities were prominently increased in the groups treated with both DMF and Tol compared with groups of single treatment with DMF. (iii) No significant changes were observed in the activities of GST and GSHPx and LPO contents. (iv) Liver function tests showed that AST, ALT, and ALP were increased by treatment with DMF and Tol and with signs of moderate and dramatic hepatic injuries in the rats of the highly exposed group (D3) and the combinatory group of DMF and Tol (DT3). The hepatotoxicities induced by DMF and by its de-methylated metabolite, NMF, have been observed in several species including rats, mice, and humans (36,37), and the hepatotoxicities were more severe by NMF than by DMF itself (8,9,38). As described in the introduction, DMF is hydroxylated at its methyl moieties by CYP to DMF-OH and NMF is generated by an enzymatic and non-enzymatic reaction (6,7), accompanying the production of reactive oxygen species (ROSs) during the degradation of DMF and NMF under aerobic conditions. Thus, ROSs and reactive intermediates cause liver toxicity, usually increasing in serum transaminases, such as AST and ALT which have been useful indicators in the evaluation of liver injury for workers occupationally exposed to DMF (39,40). ROSs are produced endogenously by normal metabolic processes, but their levels are markedly increased by environ-

mental pollutants such as chemicals, heavy metals, cigarette smoke, and ionizing radiation (41,42). ROSs possess the potential to damage either cellular or organelle membranes and macromolecules, causing lipid peroxidation, and alterations of nucleic acids and proteins. Living organisms are protected from the damages of ROS by several defence mechanisms, such as the actions of superoxide dismutase, catalase, GST, and GSHPx (43). The main function of GST is to conjugate electrophilic and hydrophobic compounds with cellular glutathione and solubilize for excretion. These reactions result in the detoxification of many endogenous electrophiles as well as in environmental chemicals (44).

In the present study, no significant change in glutathione-linked enzyme activities, such as GST and GSHPx were observed between control and the groups of animals treated with single and combined chemicals. However, the levels of CYP2E1 were considerably enhanced according to the dosages of DMF. Immunoblot densities against CYP2E1 were prominently increased in the combined group compared with the single treatment group. Also, the signs of hepatic injury appeared in the livers of the rats of the highly exposed group (D3) and the effects were dramatic in the rats treated with DMF and Tol (DT3). Thus, our results from this study suggest that CYP2E1 isozyme was inducible by DMF and Tol, and induction by Tol is responsible for the accelerated effect of the formation of NMF by induced CYP2E1 and the generated NMF might directly induce hepatotoxicity.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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