Original Article

Carbon tetrachloride-induced hepatotoxicity in pregnant and lactating rats

Masahiro Mochizuki, Satomi Shimizu, Yoshinaka Urasoko, Kazuhiko Umeshita,
Takashi Kamata, Takahiro Kitazawa, Daichi Nakamura, Yoshito Nishihata,
Takumi Ohishi and Hiroshi Edamoto

Toxicology Department, Gotemba Laboratory, Bozo Research Center Inc., 1284 Kamado, Gotemba-shi, Shizuoka
412-0039, Japan

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ABSTRACT — Carbon tetrachloride (CCl_4) is well known to induce hepatotoxicity after being metabolized to trichloromethyl free radical ($\cdot\text{CCl}_3$) by CYP2E1. In the present study, the hepatotoxicity induced by a single oral dose (2,000 mg/kg) of CCl_4 was compared between pregnant (gestation days (GD) 13 and 19) or postpartum (postpartum days (PPD) 1, 13 and 27) and non-pregnant rats. Hepatotoxicity in CCl_4 -treated pregnant rats evaluated by blood chemistry (alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activities) and histopathological finding (area of damaged hepatocytes) was minimal on GD19, being weaker than that in non-pregnant rats. CYP2E1 expression in non-treated pregnant rats decreased as pregnancy progressed and reached minimum level on GD19. Thus, the degree of CCl_4 -induced hepatotoxicity roughly corresponded to CYP2E1 levels during pregnancy. After delivery, hepatotoxicity in CCl_4 -treated lactating rats was maximal on PPD13, being stronger than that in non-pregnant rats, and then it decreased slightly on PPD27. The CYP2E1 level in the non-treated lactating rats tended to increase but remained at lower levels until PPD13 compared with that in non-pregnant rats. Thus, the degree of CCl_4 -induced hepatotoxicity did not correspond to CYP2E1 levels during lactation. This suggests that during lactation, there may be certain factors other than CYP2E1 expression responsible for the degree of CCl_4 -induced hepatotoxicity.

Key words: Carbon tetrachloride, Hepatotoxicity, Pregnancy, Lactation, CYP2E1, Rat

INTRODUCTION

During pregnancy, significant changes are observed in blood parameters in rats and rabbits as well as in women (Urasoko *et al.*, 2008; Honda *et al.*, 2008; de Rijk *et al.*, 2002; Katoh *et al.*, 1992; Wells *et al.*, 1999). In addition, it is reported that total cytochrome P450 content and CYP3A and CYP2E1 expression levels decrease in the liver of pregnant rats (Dean and Stoch, 1975; Starkel *et al.*, 2000), and that CYP2B1, CYP2B2, CYP2C6, CYP2E1 and CYP4A1 out of nine examined (CYP1A1, CYP2B1/CYP2B2, CYP2C6, CYP2C12, CYP2D1, CYP2D4, CYP2E1, CYP3A1 and CYP4A1) decrease during late pregnancy and early lactation in rats (He *et al.*, 2005a, 2005b). These findings suggest that the mode of chemical-induced toxicity may probably be modified under pregnant and lactating conditions. For exam-

ple, Carbon tetrachloride (CCl_4) is said to induce hepatic damage after being metabolized to trichloromethyl free radical ($\cdot\text{CCl}_3$) by CYP2E1 (Parkinson, 2001; Masuda, 2006), and CYP2E1 expression changes during gestation and lactation as mentioned above (He *et al.*, 2005a, 2005b). Therefore, CCl_4 -induced hepatotoxicity seems to be modified in pregnant and lactating rats. The present study was carried out to examine this possibility.

MATERIALS AND METHODS

Animals

Male and female specific pathogen-free rats of the Sprague-Dawley strain (CrI:CD(SD), Atsugi Breeding Center, Charles River Japan Inc., Kanagawa, Japan) were mated at 11 to 12 weeks of age in our laboratory, and 40 pregnant rats were obtained. In addition, 8 non-pregnant

females (14-week-old) were used as controls.

As the CCl₄-treated group, 25 pregnant rats and 5 non-pregnant rats at 14 weeks of age were used for blood chemistry and histopathological examinations. In addition, 15 out of the 25 pregnant rats and 3 out of 5 the non-pregnant rats were used for determining CYP2E1 expression. As the non-treated group, another 15 pregnant rats and 3 non-pregnant rats at 14 weeks of age were used for determining CYP2E1 expression during the pregnant and postpartum periods.

The animals were kept individually in a mesh cage in an air-conditioned animal room (temperature: $23 \pm 3^{\circ}\text{C}$; humidity: $50 \pm 20\%$; air ventilation: 10-15 times/hr; lighting: 12-hr/12-hr light/dark cycle). Animals from mating to gestation days (GD)17 and from postpartum days (PPD)21 to PPD28 were housed individually in stainless steel wire mesh cages, and the animals from GD17 to PPD21 were housed individually with their litter in plastic cages with bedding. On PPD4, litter size was reduced to 8 pups (4 males and 4 females as far as possible) by random selection, and they were weaned on PPD21. The animals were allowed free access to pellet diet (NMF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and tap water *ad libitum* during the experimental period.

Chemicals

CCl₄ and corn oil were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). CCl₄ was dissolved in corn oil immediately before use, and the concentration was adjusted to 50 vol%.

Treatments

For blood chemistry and histopathological examinations, 5 pregnant animals were orally administered a single dose (2,000 mg/kg) of CCl₄ (Nagase and Tanaka, 1976) on GD12 and 18 and PPD0, 12 and 26, respectively. Five non-pregnant rats were also given a single oral dose (2,000 mg/kg) of CCl₄ at the start of the experiment, and served as controls. Twenty-four hours after dosing, the animals were killed by exsanguination from the abdominal aorta under ether anesthesia. As mentioned above, 3 treated and 3 non-treated pregnant animals were used for determining CYP2E1 expression by Western blotting on GD13 and 19 and PPD1, 13 and 27, respectively.

All procedures on this study were conducted in compliance with "Guidelines for Proper Conduct of Animal Experiments" (Science Council of Japan, June 1, 2006) and according to the Protocol approved by the Animal Care and Use Committee at BOZO Research Center Inc.. All efforts were made to minimize animal suffering.

Blood chemical analysis

Blood samples collected from the abdominal aorta under ether anesthesia were put into tubes (test tubes containing coagulation promoting agent, Venoject II-Autosep, Terumo Corporation, Tokyo, Japan). The samples were centrifuged (approximately $1,600 \times g$, 10 min) to obtain sera, and alkaline phosphatase (ALP), triglyceride (TG), total cholesterol (T-CHO), total bilirubin (T-BIL) and phospholipids (PL) were measured using a clinical chemistry autoanalyzer, TBA-120FR (Toshiba Corporation, Tokyo, Japan).

Blood was also collected into heparinized test tubes (approximately 20 units of heparin per 1 ml blood) and centrifuged (approximately $1,600 \times g$, 10 min) to obtain plasma, and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) activities were measured using a clinical chemistry autoanalyzer, TBA-120FR.

Histopathological examination

The liver was excised from each animal at necropsy, and then fixed in phosphate-buffered 10% formalin. Four- μm paraffin sections were stained with hematoxylin and eosin (H.E) for histopathological examination.

Preparation of hepatic microsomes

Liver microsomes were prepared for 3 pregnant and non-pregnant animals each of non-treated and CCl₄-treated groups at necropsy as scheduled. The liver was excised from each animal at necropsy, perfused with 1.15% KCl solution and frozen at -80°C prior to microsomal preparation. Then the liver was thawed and homogenized with 1.15% KCl solution to achieve a 25% homogenate. The homogenate was centrifuged at $9,000 \times g$ for 20 min. S9 was also prepared and centrifuged at $105,000 \times g$ for 60 min to prepare a liver microsomal solution. Protein content was determined according to the method described by Lowry *et al.* (1951) using a spectrophotometer U-1100 (Hitachi, Ltd., Tokyo, Japan).

Electrophoresis and Western blot analysis

Protein concentration was adjusted for sodium dodecyl sulfate (SDS) / urea-polyacrylamide gel electrophoresis according to Laemmli (1970). Western blot analysis was performed using the techniques described by Kamata *et al.* (2005). Proteins were transferred onto polyvinylidene difluoride membranes (PVDF; Bio-Rad, Richmond, CA, USA) at $2 \text{ mA}/\text{cm}^2$ of constant current for 60 min and blocked for 1 hr at room temperature in 4% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan). Membranes were hybridized with a primary CYP2E1

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antibody (CHEMICON international, Inc., Temecula, CA, USA) diluted 500-times with 0.4% Block Ace overnight and then with horseradish peroxidase (HRP)-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) diluted 500-times with Tris buffered saline with Tween 20 (TTBS) for 8 hr. The membranes were incubated with 4-CN (4-chloro-1-naphthol, Wako Pure Chemical Industries, Ltd., Tokyo, Japan) to detect the proteins. Density of protein bands was calculated using the Scion (Scion Corporation, Frederick, MD, USA).

Statistical analysis

Significance of differences from the control group was tested after calculating Mean $\pm$ S.D. in each group. Homogeneity of variance was assessed by Bartlett's test (Snedecor and Cochran, 1989). For homogeneous data (level of significance: 1%, two-tailed), values were compared between the control group and each treated group by Dunnett's test (level of significance: 1 and 5%, two-tailed) (Dunnett, 1955, 1964).

RESULTS

Blood chemistry findings

Results of blood chemical analysis are shown in Table 1. In CCl₄-treated groups, ALT activity on GD13 and 19 was 40 and 4%, respectively, of that in non-pregnant animals. In addition, ALT activity on PPD1, 13 and 27 was 22, 119 and 35%, respectively, of that in non-pregnant animals. AST activity on GD13 and 19 was 63 and 13%, respectively, and that on PPD1, 13 and 27 was 101, 164 and 80%, respectively, of that in non-pregnant animals. LDH activity on GD13 and 19 was 58 and 8%, respectively, of

that in non-pregnant animals. In addition, LDH activity on PPD1, 13 and 27 was 45, 98 and 32%, respectively, of that in non-pregnant animals. As for other blood chemistry parameters examined in the CCl₄-treated groups, there were no influences of pregnancy and lactation observed compared with non-pregnant animals.

Histopathological findings

CCl₄-induced hepatic lesions were characterized by coagulation necrosis and/or vacuolation of hepatocytes mainly situated in the central to middle part of hepatic lobules (Fig. 1). In CCl₄-treated non-pregnant animals, hepatic lesions were observed from central to middle parts of the hepatic lobule. In CCl₄-treated pregnant animals, histopathological nature and degree of hepatic lesions on GD13 were equal to those in non-pregnant animals. On GD19, hepatic lesions were prominently reduced and limited to the central part of hepatic lobules. Hepatic lesions tended to increase again after delivery and became more marked than those in non-pregnant animals on PPD13. On PPD27, hepatic lesions, however, returned to almost the same degree as those in non-pregnant rats.

Findings of Western blot analysis

Results of Western blot analysis in non-treated groups are shown in Figs. 2 and 3. CYP2E1 expression in non-treated groups decreased as pregnancy progressed but it tended to increase after delivery. Specifically, CYP2E1 expression in pregnant animals on GD13 and GD19 was 68 and 30% of that in non-pregnant animals, respectively, and CYP2E1 expression in postpartum animals on PPD1, PPD13 and PPD27 was 72, 78 and 112% of that in non-pregnant animals, respectively.

On the other hand, in CCl₄-treated groups, CYP2E1

Table 1. Effects caused by single administration of CCl₄ on blood chemistry parameters of pregnant and postpartum rats

Parameter/Stage	Control	GD13	GD19	PPD1	PPD13	PPD27
ALT (IU/l)	3968 $\pm$ 3409	1568 $\pm$ 1833	156 $\pm$ 162*	872 $\pm$ 799	4720 $\pm$ 3525	1400 $\pm$ 901
AST (IU/l)	11224 $\pm$ 6246	7092 $\pm$ 6184	1425 $\pm$ 1970	11328 $\pm$ 11613	18444 $\pm$ 8665	9012 $\pm$ 5227
LDH (IU/l)	21556 $\pm$ 21159	12564 $\pm$ 14562	1650 $\pm$ 3262	9756 $\pm$ 15645	21164 $\pm$ 18881	6792 $\pm$ 5552
ALP (IU/l)	533 $\pm$ 72	586 $\pm$ 129	305 $\pm$ 96	337 $\pm$ 156	843 $\pm$ 392	551 $\pm$ 151
TG (mg/dl)	50 $\pm$ 22	66 $\pm$ 71	443 $\pm$ 368**	86 $\pm$ 85	112 $\pm$ 88	48 $\pm$ 18
T-CHO (mg/dl)	47 $\pm$ 10	30 $\pm$ 18	63 $\pm$ 24	55 $\pm$ 10	74 $\pm$ 14	44 $\pm$ 17
PL (mg/dl)	91 $\pm$ 18	60 $\pm$ 25	154 $\pm$ 70	107 $\pm$ 18	136 $\pm$ 42	90 $\pm$ 24
T-BIL (mg/dl)	1.6 $\pm$ 1.5	0.28 $\pm$ 0.3	0.1 $\pm$ 0.1*	0.3 $\pm$ 0.2	1.4 $\pm$ 1.3	0.2 $\pm$ 0

The CCl₄ level was set at 2,000 mg/kg.

Each value represents the mean $\pm$ S.D. of 5 female rats.

* p < 0.05, ** p < 0.01: Significantly different from the control group mean (Dunnett's test).

Control: non-pregnant rats, GD 13 and 19: pregnant rats, PPD 1 and 13: lactating rats,

PPD27: post-lactating rats.

Fig. 1. Histopathological examination in the liver at 24 hr after dosing of CCl_4 . Hematoxylin and eosin staining, magnification $\times 100$. Control: non-pregnant rats, GD 13 and 19: pregnant rats, PPD 1 and 13: lactating rats, PPD27: post-lactating rats. (scale bar = $100\mu\text{m}$)

expression in pregnant animals on GD13 and GD19 was 0.3 and 7%, respectively, and that in postpartum animals on PPD1, PPD13 and PPD27 was 0.7, 5 and 0.4%, respectively, compared with that in non-pregnant animals.

DISCUSSION

In this study, CCl_4 -induced hepatotoxicity was compared between pregnant or postpartum and non-pregnant rats.

Activities of AST, ALT and LDH as well as the extent of hepatic lesions were prominently reduced on GD19 when CYP2E1 expression showed the lowest level. Thus, the degree of CCl_4 -induced hepatotoxicity in pregnant animals seemed to roughly correspond to the expression level of CYP2E1 during pregnancy. CYP2E1 expression levels observed in the present study decreased along with the progression of pregnancy as reported previously (He *et al.*, 2005a; Starkel *et al.*, 2000). Dean and Stock (1975) suggested that lower levels of hepatic microsomal enzyme activity might reflect a biological control mechanism to ensure elevated levels of progesterone required to maintain pregnancy. Oxidative stress is said to decrease total CYP450 levels and drug-metabolizing activities *in vivo* (Liu *et al.*, 1993), and He *et al.* (2005a) suggested that down-regulation of CYP2E1 levels during pregnan-

cy observed in their study might be due to increased oxidative stress during pregnancy. Pahan *et al.* (1997) also demonstrated down-regulation of CYP2E1 expression in rat liver peroxisomes by ischemia/reperfusion-induced oxidative stress. In addition, it is known that endogenous nitric oxide (NO) production increases to maintain blood flow during pregnancy (Xu *et al.*, 1996), and that endogenous NO also inhibits CYPs expression (Gonzalez, 1990; Khatsenko *et al.*, 1997; Minamiyama *et al.*, 1997). On the other hand, it has been documented that lipid peroxidation remains low until midpregnancy and begins to increase after GD15 in rats (Sugino *et al.*, 1993), and as a protective factor against lipid peroxidation, superoxide dismutase (SOD) slightly increases in the liver of pregnant rats, but catalase and glutathione peroxidase (GSH-Px) significantly decrease (Yoshioka, *et al.*, 1987). In addition, Mover-Lev and Ar (1997) reported a decrease in GSH-Px in the liver and placenta of pregnant rats, and they suggested that oxidative stress might increase during pregnancy. These findings are considered to be attributable to the decreased CYP2E1 expression in pregnant rats observed in the present study.

Activities of ALT, AST and LDH as well as degree of hepatic lesions which decreased on GD19 compared with those in non-pregnant animals tended to be restored after delivery, and, on PPD13, activities of the above-men-

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Fig. 2. CYP2E1 expressions by western blot analysis of liver microsomes from non-pregnant, pregnant, lactating and post-lactating rats.

Control: non-pregnant control, GD 13 and 19: pregnant rats, PPD 1 and 13: lactating rats, PPD27: post-lactating rats.

Fig. 3. The density of Western blotting using monoclonal antibodies against rat hepatic CYP2E1.

Each value represents the mean of three rats.

**p < 0.01: Significantly different from the control group mean (Dunnett's test).

Control: non-pregnant rats, GD 13 and 19: pregnant rats, PPD 1 and 13: lactating rats, PPD27: post-lactating rats.

tioned enzymes were equal to or higher than those in non-pregnant animals. In addition, the degree of hepatic lesions was more marked than in non-pregnant animals. After that, the activities of the above-mentioned enzymes were lower than those in non-pregnant animals and the degree of hepatic lesions was equal to that in non-pregnant animals on PPD27. As to CYP2E1 expression, which tended to be restored after delivery, the levels still remained lower than that in non-pregnant animals until PPD13, and then returned to the level of non-pregnant animals on PPD27. Thus the degree of CCl₄-induced hepatotoxicity did not correspond to the expression level of CYP2E1 after delivery. Papworth and Clubb (1995) reported that ALT, AST and ALP activities increased during the lactation period of rats, and they suggested that such changes might reflect an increase in hepatic metab-

olism of dams in order to adapt themselves to increased food consumption/milk production for their pups. In addition, a study in ewes by El-Sherif and Assad (2001) showed that increases in AST and ALT activities reflected a state of hyperfunction during the lactation period, and another study in goats by Sarma and Ray (1985) showed that AST activity was high post partum and was positively correlated with milk yield. Moreover, Toropila *et al.* (2005) reported that activities of adaptive enzymes, ALT and AST, increased in hepatocytes of fasting rats due to endogenous synthesis of energy. Thus, the metabolic load on the liver increases in lactating animals compared with non-pregnant animals, and this may influence the mode of CCl₄-induced hepatotoxicity during lactation.

GSH transportation to the mammary gland is essential to keep lactation, and the GSH released from the liver

in lactating rats was higher than that in non-pregnant rats (Barber *et al.*, 1999). On the other hand, decreased GSH-release led to apoptosis and involution of the mammary gland, and GSH synthesis in the liver decreased after the peak of lactation (PPD12 to 15) (Zaragoza *et al.*, 2003, 2005). In addition, lipid peroxidation levels significantly increased in all major rat organs during the lactation period (Upreti *et al.*, 2002). Therefore, changes in GSH and oxidative stress may also have a certain relation to the changes in CCl₄-induced hepatotoxicity during lactation.

In conclusion, the present results strongly suggest that the mode of CCl₄-induced hepatotoxicity in rats may be influenced by pregnancy and lactation.

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