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Original Article

Carbamazepine-induced liver injury using type 2 diabetes Spontaneously Diabetic Torii-*Lepr*^{fa} (SDT fatty) rats as a model for human type 2 diabetes

Tadakazu Takahashi[1,2], Chizuru Matsuura[1], Kaoru Toyoda[1], Yusuke Suzuki[1], Naohito Yamada[1], Akio Kobayashi[1], Shoichiro Sugai[1] and Kayoko Shimoi[2]

[1]Toxicology Research Lab., Central Pharmaceutical Research Institute, JAPAN TOBACCO INC., 1-13-2 Fukuura, Kanazawa, Yokohama, Kanagawa 236-0004, Japan
[2]Graduate School of Integrated Pharmaceutical and Nutritional Sciences, Graduate Program in Environmental Health Sciences, University of Shizuoka

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ABSTRACT — The diabetic state is considered to be one of the risk factors of drug-induced liver injury (DILI) because of the lower levels of glutathione for detoxification by conjugation with drugs. Carbamazepine (CBZ) -induced hepatotoxicity in humans is rare and unpredictable with the present state of knowledge, but it is somehow related to disturbance of glutathione metabolism, although data in this regard are limited. In order to estimate the potential risk of DILI in patients with type 2 diabetes mellitus (T2DM), we investigated the liver injury from CBZ, which is often used in the treatment of painful diabetic neuropathy in diabetic patients, using SD rats and Spontaneously Diabetic Torii-*Lepr*^{fa} (SDT fatty) rats as a model for human T2DM. The SDT fatty rats appropriately mimic the diabetic state in humans and have similar profiles of glucose metabolism, hepatic function tests and glutathione synthesis to those in patients with T2DM. Short-term oral dosing with CBZ to the SDT fatty rats revealed that liver injury was detected in the SDT fatty rats but not in the SD rats and the difference was considered to be due to lower hepatic detoxification of the metabolites of CBZ by depleted hepatic glutathione synthesis. In conclusion, the potential for CBZ to induce liver injury is considered to be higher in diabetic patients than in non-diabetic humans.

Key words: SDT fatty rats, Type2 diabetes, Hepatotoxicity, Glutathione, Carbamazepine

INTRODUCTION

Drug-induced liver injury (DILI) is considered to be an important matter for the public health including its potential impact on the development of new drugs. DILI events are also the main cause of regulatory action pertaining to drugs, including denial of marketing approval, restrictions with respect to clinical indications and withdrawal from the marketplace (Lee, 2003; Smith and Schmid, 2006).

Carbamazepine (CBZ) suppresses the spread of seizure activity by reduction in the post-tetanic potentiation of synaptic transmission (the Clinical and Research Information on DILI by NIH in the U.S.). CBZ was approved for use in epilepsy in the United States in 1968 and it is still in common use with more than 2 million prescriptions being written yearly. CBZ is also used clinically for the treatment of painful diabetic neuropathy (PDN) which is the most common microvascular complication of diabetes mellitus with peripheral nerve dysfunction (Saeed *et al.*, 2014). Clinically apparent hepatotoxicity from

Correspondence: Tadakazu Takahashi (E-mail: tadakazu.takahashi@jt.com)

CBZ is uncommon but well described, there being several hundred cases reported in the literature (the Clinical and Research Information on DILI by NIH in the U.S.). In addition, a transient and asymptomatic elevation of liver enzymes occurs in 25-61% of patients receiving CBZ (Benedetti *et al.*, 2005). The most common pattern of enzyme elevations in CBZ related drug effects include rashes with eosinophilia and systemic symptoms such as DRESS syndrome, a mixed or cholestatic injury. Liver biopsy shows the cholestatic injury with focal hepatocellular necrosis, prominence of eosinophils and occasionally granulomas (the Clinical and Research Information on DILI by NIH in the U.S.). The metabolism of CBZ is thought to play an important role in the pathogenesis of CBZ hypersensitivity and hepatotoxicity and it has been postulated that metabolites of CBZ are causative agents (Pandit *et al.*, 2012). The development of CBZ-induced hepatotoxicity is somehow related to disturbance of glutathione metabolism, although data in this regard are limited (Kalapos, 2002). CBZ is metabolized by the hepatic CYP3A family of microsomal enzymes and epoxidation and hydroxylation are the main metabolic pathways, though conjugation reactions may also play a role (Bernus *et al.*, 1996; Maheswari *et al.*, 2014). The arene oxide metabolite of CBZ may lead to hapten formation and via the immune system results in the tissue injury at the sites of hapten formation, including the liver (Leeder and Pirmohamad, 2003). In addition, reactive metabolite(s) of CBZ produced by CYP3A under GSH-depleted conditions might be involved in the development of liver injury in rats (Iida *et al.*, 2015).

Disturbances in glutathione (GSH) homeostasis have been associated with liver diseases induced by drugs because glutathione is the most abundant cellular thiol antioxidant (Chen *et al.*, 2013). It has been reported that one of the potential risks of drug induced liver injury related to acetaminophen in humans is a lower level of glutathione (Sun *et al.,* 2009). Taking into account that reactive metabolites, which are often the cause of DILI, are detoxified by conjugation with glutathione, Sun's observation is reasonable. Diabetic patients have decreased levels of glutathione in the blood possibly due to compromised levels of GSH synthesis and metabolism enzymes (Illing *et al.*, 1951; Lal and Kumar, 1967; Awadallah *et al.*, 1978; Lagman *et al*., 2015). From these observations, the risk of DILI is considered to be higher in diabetic patients than in nondiabetic humans for drugs which generate reactive metabolites. Thus, estimation of the potential risk of hepatotoxicity, induced by drugs which are detoxified by glutathione, is considered to be important to improve the safety of the diabetic patients.

Spontaneously Diabetic Torii-*Lepr*^{fa} (SDT fatty) rats is a model of obese type 2 diabetes in which the *fa* allele of the Zucker fatty rat was introduced into the Spontaneously Diabetic Torii (SDT) rat genome (Shinohara *et al.*, 2000; Masuyama *et al*., 2005). The male SDT fatty rats have overt obesity, and hyperglycemia and hyperlipidemia are observed from four to six weeks of age (Matsui *et al*., 2008). It has been reported that the SDT fatty rats shows the pathological profile of diabetic complications including diabetic nephropathy, cataracts, retinal findings and osteoporosis (Matsui *et al*., 2008; Katsuda *et al.*, 2014). The following profiles in the SDT fatty rats are similar to those in T2DM patients: glucose metabolism (accelerated hepatic gluconeogenesis and fatty acid β-oxidation, decelerated glycolysis), hepatic function (higher plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (GGT) and glutamate dehydrogenase (GLDH) without liver injury) and decreased hepatic glutathione synthesis (lower hepatic reduced- and oxidized-form glutathione levels) (Takahashi *et al.*, 2019). In addition, our previous investigations revealed that allyl alcohol induced liver injury was markedly enhanced in the SDT fatty rats after five-week repeated oral dosing of allyl alcohol when compared with SD rats and the difference was considered to be due to lower hepatic detoxification of acrolein, the reactive metabolite of allyl alcohol, due to depleted hepatic glutathione synthesis (Takahashi *et al.*, 2019). Thus, in the present study, we investigated CBZ-induced liver injury in SDT fatty rats for estimation of potential risk of liver injury in diabetic patients.

MATERIALS AND METHODS

Animals

Sixteen male Jcl:SD (SD) rats and twenty-one male SDT.Cg-*Lepr*^{fa}/JttJcl (SDT fatty) rats at 12 and 5 weeks of age, respectively, were obtained from CLEA Japan Inc. (Tokyo, Japan). The animals were housed individually in wire-mesh cages kept in an air-conditioned room with a 12-hr light-dark cycle (lighting from 7:00 a.m. to 7:00 p.m.) at a temperature of 23 ± 1°C, a relative humidity of 55 ± 5% and a ventilation rate of about 15 times per hour. The SD and SDT fatty rats were quarantined and acclimated for 1 and 8 weeks, respectively, and were allowed free access to a commercial pelleted diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) *ad libitum* during the quarantine/acclimated and dosing periods. Tap water was available for drinking *ad libitum*. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Toxicology

Research Laboratories, Central Pharmaceutical Research Institute, Japan Tobacco Inc. This study was conducted in accordance with the Japanese Law for the Humane Treatment and Management of Animals (Law No. 105, as revised in 2013, issued in October 1, 1973).

Dosing of carbamazepine

CBZ was purchased from Accela ChemBio Co., Ltd. (San Diego, CA, USA) and was suspended in 0.5% methylcellulose (MC, Shin-etsu Chemical Co., Ltd., Tokyo, Japan) aqueous solution. Each strain of rats was randomly allocated to each group based on the day before the initiation of dosing so that the initial mean body weights of each group were equivalent. CBZ was administered orally once daily to the SD and SDT fatty rats (13 weeks of age, 8 rats per group for the SD rats and 10 or 11 rats per group for the SDT fatty rats) at the dose levels of 0 (vehicle control for each strain) and 400 mg/kg for three to five days. The dose level of 400 mg/kg was selected for both the SD and SDT fatty rats as the expected dose level to induce hepatotoxicity (increases in the plasma parameters for hepatic function and hepatocellular damage) based on a study with glutathione-depleted rats treated with L-Buthionine (S,R)-sulfoximine (BSO) (Iida *et al.*, 2015). The animals in the matched control groups for each strain of rats were given the vehicle (0.5% MC aqueous solution). The dosing volume was set at 5.0 mL/kg and was calculated based on the most recently recorded body weights during the dosing period.

Observations, measurements and examinations

Clinical observations, measurements of body weights and food consumption

The animals were observed carefully for any clinical signs twice daily (before and after daily dosing) during the dosing period. The animals were weighed and food consumption per animal was calculated every day during the dosing period.

Blood and liver sampling

At the end of the dosing period (day 6: the day after the last dose), the animals' abdomens were opened under isoflurane anesthesia and blood samples were taken from the abdominal aorta under the fed condition. Plasma samples, obtained as described previously (Kondo *et al*., 2012), were used for the measurement of the plasma parameters related to the hepatic function and hepatobiliary system including AST, ALT, guanase (GU), alkaline phosphatase (ALP) and total bilirubin (T-BIL). Then, samples of the liver were taken from all the SD and SDT fatty rats in the control and CBZ-treated groups and frozen with liquid nitrogen. The liver samples were stored frozen in an ultra-deep freezer set at –80°C until use.

Measurements of plasma parameters for hepatic function

Plasma AST, ALT, GU and ALP activities were measured at 37°C with a TBA-120FR automated analyzer using standard reagents by UV kinetic method for AST, ALT and ALP (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and GU (Serotec Co., Ltd., Hokkaido, Japan). Plasma T-BIL concentrations were measured at 37°C with a TBA-120FR automated analyzer using standard reagents by enzymatic method for these parameters (KANTO CHEMICAL CO., INC., Tokyo, Japan).

Measurements of the liver weights

At the terminal sacrifice, the final body weights from all the animals were recorded to calculate the relative weights of the liver to the body weights. The livers were weighed and the relative weights of the liver to the final body weights were calculated.

Assay of hepatic reduced glutathione and oxidized glutathione levels

A liver sample of approximately 1 g was collected from each animal. Preparation of the samples and measurement of the hepatic reduced glutathione (GSH) and oxidized glutathione (GSSG) concentrations were performed according to the previous description (Takahashi *et al.*, 2019) using GSH/GSSG quantification kit (Dojindo laboratories, Kumamoto, Japan).

Assay of hepatic lipid peroxide levels

The frozen liver samples were thawed and PBS (TAKARA BIO INC., Shiga, Japan) containing 0.1 wt% Triton X-100 (F. Hoffmann-La Roche, Ltd., Basel, Switzerland) and 0.05 wt% sodium deoxycholate (FUJIFILM Wako Pure Chemical Corporation) was added to the samples at a volume of 4 mL per 1 g wet tissue weight. Preparation of the samples and measurement of the hepatic lipid peroxide (LPO) concentrations were performed according to the previous description (Takahashi, *et al.*, 2019) using a colorimetric method using LPO-CC kit (KAMIYA BIOMEDICAL COMPANY, Seattle, WA, USA).

Histopathological examination in the liver

The left lobe of the liver was cut into longitudinal sections and the liver slices were embedded in paraffin. Sectioning and hematoxylin-eosin staining was performed according to routine histological procedures.

Statistical analysis

The mean values and standard deviations in each group were calculated for the body weights, food consumption, plasma parameters for hepatic function, the liver weights, hepatic GSH and GSSG concentrations and hepatic LPO concentrations at the end of the dosing period. A Student's t test was conducted for comparison of the parameters mentioned above between the control SD and SDT fatty rats and between the vehicle control and CBZ-treated groups each of the SD and SDT fatty rats. The levels of significance were set at 5% and 1% (one- or two-tailed).

RESULTS

To confirm the hepatic function, the hepatic glutathione and oxidative stress levels in the SDT fatty rats employed in this study, the following parameters were compared between the control SD and SDT fatty rats. At 13 weeks of age, the SDT fatty rats had higher values of body weights and food consumption (Fig. 1) ($p < 0.01$), higher levels of plasma AST, ALT and GU (Tables 1 and 2) ($p < 0.05$ or $p < 0.01$), higher liver weights (Tables 1 and 2) ($p < 0.01$), lower hepatic GSH and GSSG levels and higher LPO levels when compared with the SD rats (Fig. 2)

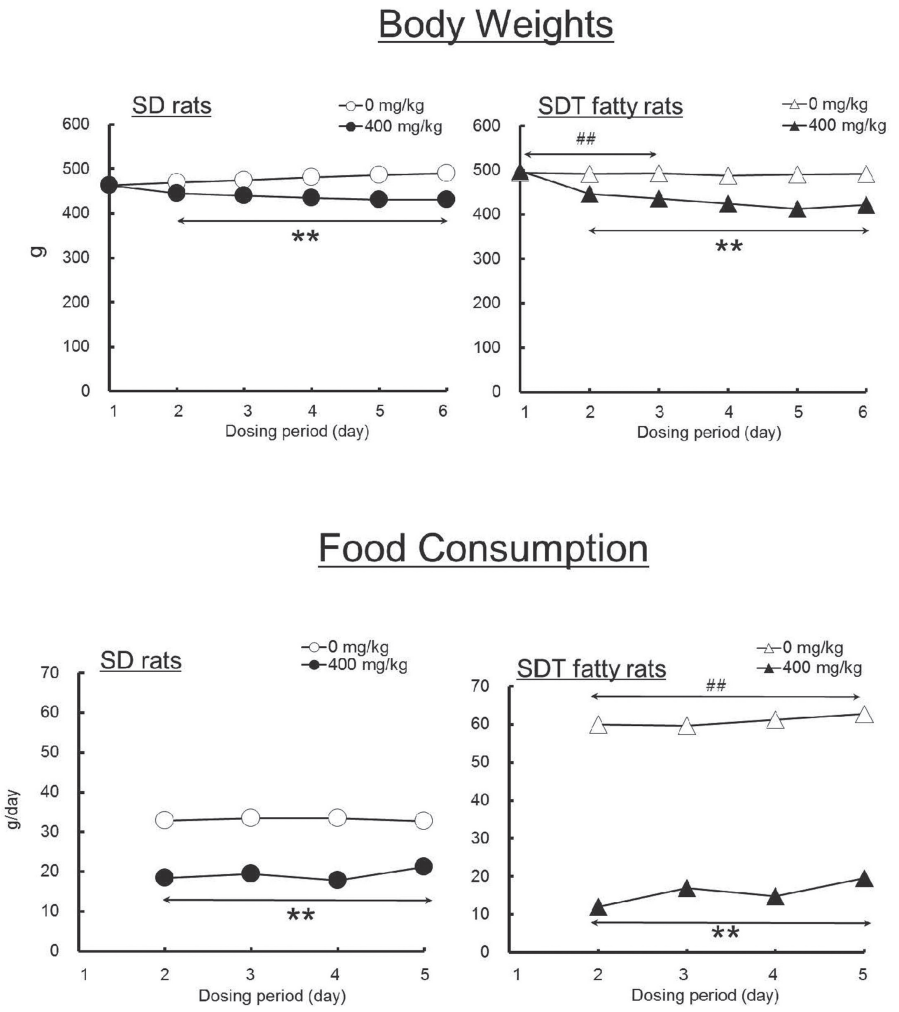

Fig. 1. Body weights and food consumption in the CBZ-treated SD and SDT fatty rats during the dosing period. All the CBZ-treated SDT fatty rats were withdrawn from the drug on day 4 and four animals (Animal Nos., 30, 33, 35 and 36) were subsequently withdrawn from the drug on day 5 because of the deterioration of the animals' physical condition. Mean values are shown in the figure. ## < 0.01 significantly different between the control SD and SDT fatty rats (Student's *t*-test), ** $p < 0.01$ significantly different from the matched control group (Student's *t*-test)

Table 1. Individual data of plasma parameters for hepatic function, the liver weights and the histopathological examinations of the liver in the control and CBZ-treated SD rats.

Strain: SD rats										
Dose Levels of CBZ:	0 mg/kg									
Animal No.:	1	2	3	4	5	6	7	8	Mean ± S.D.	
Hepatic Function Parameters in Plasma										
AST (U/L)	56	64	60	65	56	58	62	62	60.4 ± 3.5	
ALT (U/L)	26	32	37	37	23	24	25	31	29.4 ± 5.7	
GU (U/L)	25	26	21	24	24	23	23	22	23.5 ± 1.6	
ALP (U/L)	671	597	515	821	740	803	683	693	690.4 ± 101.5	
T-BIL (mg/dL)	0.07	0.05	0.06	0.05	0.05	0.05	0.06	0.07	0.058 ± 0.009	
Liver Weights										
Absolute weights (g)	17.52	16.47	15.06	14.94	15.23	16.04	16.03	15.16	15.81 ± 0.890	
Relative weights (g/100 g B.W.)	3.36	3.33	3.00	3.20	3.11	3.34	3.27	3.20	3.226 ± 0.125	
Histopathological Findings in the Liver										
Necrosis, hepatocytes, centrilobular	--	--	--	--	--	--	--	--		
Hypertrophy, hepatocytes, centrilobular	--	--	--	--	--	--	--	--		
Fatty change, hepatocytes, periportal	--	--	--	--	--	--	--	--		
Deposition, glycogen, hepatocytes	+	+	±	+	+	+	+	+		
Dose Levels of CBZ:	400 mg/kg									
Animal No.:	9	10	11[$]	12	13	14	15	16	Mean ± S.D.	
Hepatic Function Parameters in Plasma										
AST (U/L)	52	81	NE	48	58	63	44	43	55.6 ± 13.4	
ALT (U/L)	33	64	NE	30	44	47	33	23	39.1 ± 13.7	*
GU (U/L)	20	27	NE	17	24	23	18	13	20.3 ± 4.8	*
ALP (U/L)	518	534	NE	572	486	556	332	312	472.9 ± 106.8	**
T-BIL (mg/dL)	0.07	0.06	NE	0.06	0.04	0.05	0.05	0.1	0.061 ± 0.020	
Liver Weights										
Absolute weights (g)	18.38	19.22	NE	19.65	18.29	18.55	14.94	14.94	17.710 ± 1.953	*
Relative weights (g/100 g B.W.)	4.16	4.23	NE	4.40	4.36	4.33	3.60	3.61	4.099 ± 0.347	**
Histopathological Findings in the Liver										
Necrosis, hepatocytes, centrilobular	--	--	--	--	--	--	--	--		
Hypertrophy, hepatocytes, centrilobular	+	±	--	+	+	+	+	±		
Fatty change, hepatocytes, periportal	--	--	--	±	--	--	--	--		
Deposition, glycogen, hepatocytes	±	±	--	±	±	±	--	--		

Abbreviation: CBZ; carbamazepine, AST; aspartate aminotransferase, ALT; alanine aminotransferase, GU; guanase, ALP; alkaline phosphatase, T-BIL; total bilirubin, S.D.: standard deviation, $: one animal (Animal No. 11) was found dead on day 1 of the dosing period. Blood samples were collected at 9:00-10:00 a.m. from each rat at the end of the dosing period (day 6) (n = 7 or 8/group) under the non-fasted conditions. *$p < 0.05$, **$p < 0.01$ significantly different from the matched control group (Student's *t*-test). Grade for the histopathological findings: --: no finding, ±: very slight, +: slight

($p < 0.01$).

To compare the effects of CBZ on the liver between the SD rats and SDT fatty rats, the following examinations were conducted for the CBZ-treated SD and SDT fatty rats.

In the CBZ-treated SD rats, one animal (Animal No. 11) was found dead on day 1 of the dosing period. Weakness of the muscle tone, abnormal gait, decreased locomotor activity and prone position were noted in all the CBZ-treated SD rats within 1 hour after dosing from

Table 2. Individual data of plasma parameters for hepatic function, the liver weights and the histopathological examinations of the liver in the control and CBZ-treated SDT fatty rats

Strain: SDT fatty rats												
Dose Levels of CBZ:	0 mg/kg											
Animal No.:	17	18	19	20	21	22	23	24	25	26	Mean ± S.D.	
Hepatic Function Parameters in Plasma												
AST (U/L)	63	50	76	130	143	62	115	89	64	121	91.3 ± 33.3	##
ALT (U/L)	102	292	116	175	197	94	156	123	115	170	154.0 ± 59.5	##
GU (U/L)	26	30	25	25	27	22	26	23	23	27	25.4 ± 2.4	#
ALP (U/L)	920	630	740	1911	500	1520	590	510	720	2770	1081.1 ± 753.9	
T-BIL (mg/dL)	0.08	0.06	0.06	0.07	0.05	0.07	0.06	0.06	0.06	0.05	0.062 ± 0.009	
Liver Weights												
Absolute weights (g)	21.24	22.99	22.26	21.79	23.21	19.7	23.12	23.6	23.89	20.56	22.236 ± 1.388	##
Relative weights (g/100 g B.W.)	4.39	4.72	4.46	4.39	4.71	4.49	4.80	4.56	4.42	4.28	4.522 ± 0.171	##
Histopathological Findings in the Liver												
Necrosis, hepatocytes, centrilobular	--	--	--	--	--	--	--	--	--	--		
Hypertrophy, hepatocytes, centrilobular	--	--	--	--	--	--	--	--	--	--		
Fatty change, hepatocytes, periportal	--	--	--	--	--	--	--	--	--	--		
Deposition, glycogen, hepatocytes	+	+	+	+	+	+	+	+	+	+		

Dose Levels of CBZ:	400 mg/kg												
Animal No.:	27	28	29	30$	31$	32	33	34	35$	36	37	Mean ± S.D.	
Hepatic Function Parameters in Plasma													
AST (U/L)	46	71	60	NE	NE	80	50	44	NE	67	46	58.0 ± 13.6	**
ALT (U/L)	77	108	84	NE	NE	146	***354***	74	NE	91	78	126.5 ± 94.9	
GU (U/L)	22	23	***36***	NE	NE	***37***	***82***	***47***	NE	***42***	21	38.8 ± 20.0	
ALP (U/L)	490	150	1287	NE	NE	1713	1755	871	NE	731	660	957.1 ± 577.3	
T-BIL (mg/dL)	0.05	0.05	0.1	NE	NE	0.06	***0.5***	0.11	NE	0.11	0.03	0.131 ± 0.168	
Liver Weights													
Absolute weights (g)	27.94	25.38	21.32	NE	NE	26.50	23.90	21.85	NE	18.30	31.88	24.634 ± 4.257	
Relative weights (g/100 g B.W.)	5.98	5.71	5.29	NE	NE	6.31	5.92	5.60	NE	5.42	6.31	5.818 ± 0.381	**
Histopathological Findings in the Liver													
Necrosis, hepatocytes, centrilobular	--	--	--	--	+	--	--	+	+	--	--		
Hypertrophy, hepatocytes, centrilobular	+	+	+	--	--	+	±	+	--	+	+		
Fatty change, hepatocytes, periportal	--	--	--	--	--	--	2+	±	--	±	--		
Deposition, glycogen, hepatocytes	±	+	--	--	--	±	--	--	--	--	+		

Abbreviation: CBZ; carbamazepine, AST; aspartate aminotransferase, ALT; alanine aminotransferase, GU; guanase, ALP; alkaline phosphatase, T-BIL; total bilirubin, S.D.: standard deviation, $: three animals (Animal Nos. 30, 31 and 35) were found dead on day 5 or 6. Blood samples were collected at 9:00-10:00 a.m. from each rat at the end of the dosing period (day 6) (n = 8 or 10/group) under non-fasted conditions. All the CBZ-treated SDT fatty rats were withdrawn from the drug on day 4 because of the deterioration of the animals' physical condition. One animal (Animal No. 31) was found dead on the next morning (day 5). The surviving six animals (Animal Nos. 27, 28, 29, 32, 34 and 37) were given CBZ on day 5 but four animals (Animal Nos. 30, 33, 35 and 36) were subsequently withdrawn from the drug on day 5 because of the deterioration of the animals' physical condition. Two animals (Animal Nos. 30 and 35) were found dead on day 6. # $p < 0.05$, ## < 0.01 significantly different between the control SD and SDT fatty rats (Student's *t*-test), ** $p < 0.01$ significantly different from the matched control group (Student's *t*-test). The individual values highlighted as bold and italic text in plasma levels of ALT, GU and T-BIL in the 400 mg/kg group were higher than those in the control group. Grade for the histopathological findings: --: no finding, ±: very slight, +: slight, 2+: moderate

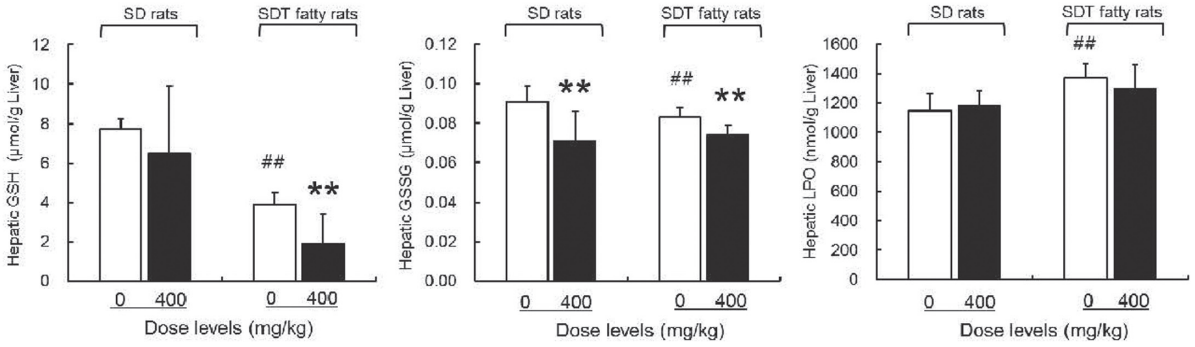

Fig. 2. Hepatic GSH, GSSG and LPO levels in the CBZ-treated SD and SDT fatty rats. Abbreviation: GSH; reduced glutathione, GSSG; oxidized glutathione, LPO; lipid peroxide. The liver samples were obtained from each rat at the end of the dosing period (day 6) (n = 7 to 10/group) under non-fasted conditions. All the CBZ-treated SDT fatty rats were withdrawn from the drug on day 4 and four animals (Animal Nos., 30, 33, 35 and 36) were subsequently withdrawn from the drug on day 5 because of the deterioration of the animals' physical condition. ## < 0.01 significantly different between the control SD and SDT fatty rats (Student's *t*-test), ** $p < 0.01$ significantly different from the matched control group (Student *t*-test)

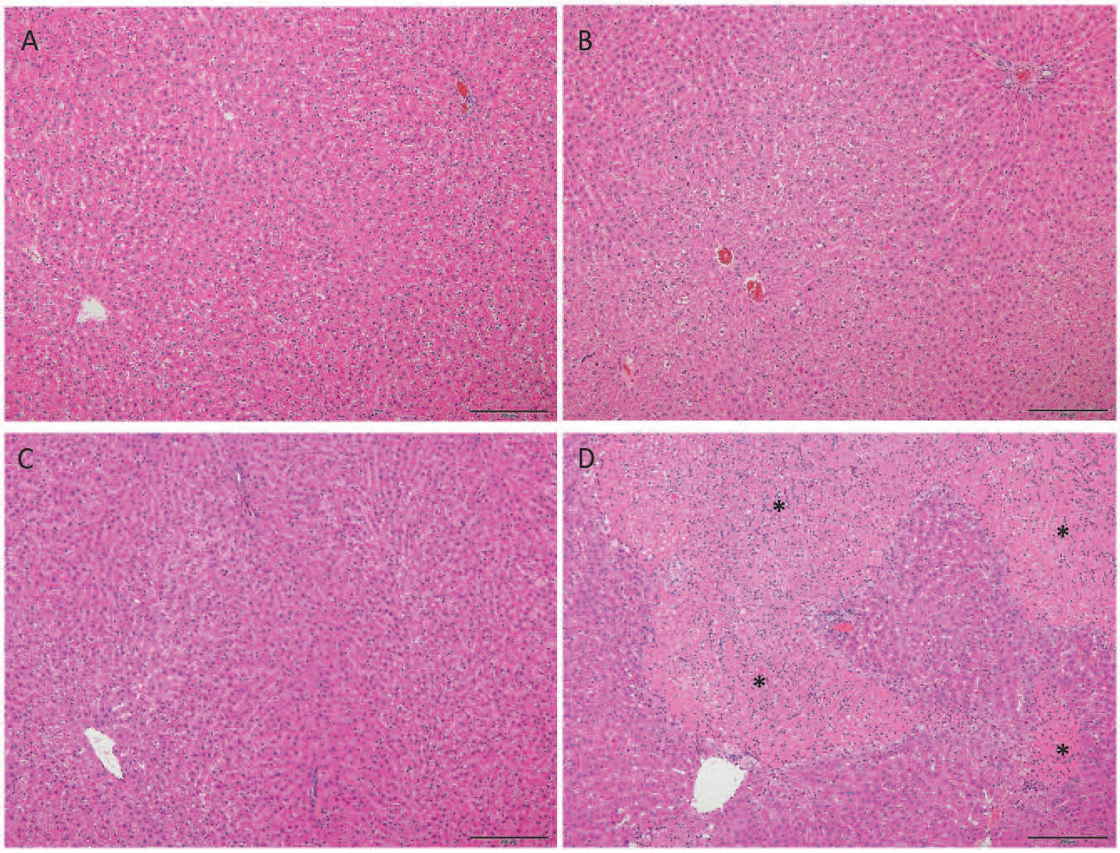

Fig. 3. Light micrographs of the liver sections in the CBZ-treated SD and SDT fatty rats. Light micrographs of hematoxylin-eosin stained liver sections from SD rat in the control group (A), SD rat in the CBZ-treated group (B), SDT fatty rat in the control group (C) and SDT fatty rat in the CBZ-treated group (D) (scale bar: 200 μm). In the CBZ-treated SDT fatty rat, necrosis of the centrilobular hepatocytes (*) were observed.

days 1 to 5. Body weights and food consumption were decreased in the CBZ-treated SD rats during the dosing period (Fig. 1) ($p < 0.01$). Plasma ALT levels were slightly increased in the CBZ-treated SD rats (Table 1) ($p < 0.05$). There were no treatment-related increases in plasma GU or ALP levels in the CBZ-treated SD rats (Table 1). The absolute liver weights and the relative weights of the liver to the body weights were increased and hepatic GSSG levels were decreased in the CBZ-treated SD rats (Fig. 2 and Table 1) ($p < 0.01$). No treatment-related changes were noted in the following parameters in the CBZ-treated SD rats when compared with the matched control group: plasma levels of AST or T-BIL, or hepatic GSH or LPO levels (Fig. 2 and Table 1). The following treatment-related histopathological findings were observed in the liver in the CBZ-treated SD rats: very slight to slight hypertrophy in the centrilobular hepatocytes, very slight fatty change in the periportal hepatocytes (only in one male) and decreased glycogen deposition in the hepatocytes (Table 1). The light micrographs of the liver sections from the SD rats in the control and CBZ-treated groups were shown in Fig. 3.

In the CBZ-treated SDT fatty rats, weakness of the muscle tone, abnormal gait, decreased locomotor activity and prone position were noted in all the CBZ-treated SDT fatty rats within 1 hr after dosing from days 1 to 3. All the CBZ-treated SDT fatty rats were withdrawn from the drug on day 4 because of the deterioration of the animals' physical condition. One animal (Animal No. 31) was found dead on the next morning (day 5). The surviving six animals (Animal Nos. 27, 28, 29, 32, 34 and 37) were given CBZ on day 5 but remaining four animals (Animal Nos. 30, 33, 35 and 36) were subsequently withdrawn from the drug on day 5 because of the deterioration of the animals' physical condition. Two animals (Animal Nos. 30 and 35) were found dead on day 6. Body weights and food consumption were decreased in the CBZ-treated SDT fatty rats during the dosing period (Fig. 1) ($p < 0.01$). Plasma GU levels tended to be higher in some animals (Animal Nos. 29, 32, 33, 34 and 36) in the CBZ-treated SDT fatty rats than those in the matched control group (Table 2). Plasma ALT and T-BIL levels also tended to be higher in one animal (Animal No. 33) than those in the matched control group (Table 2). There were no treatment-related increases in plasma AST and ALP levels in the CBZ-treated SDT fatty rats (Table 2). The relative weights of the liver to the body weights were increased and hepatic GSH and GSSG levels were decreased in the CBZ-treated SDT fatty rats when compared with the matched control group (Fig. 2 and Table 2) ($p < 0.01$). Hepatic LPO levels in the CBZ-treated SDT fatty rats were comparable to those in the matched control group (Fig. 2). The following treatment-related histopathological findings were observed in the liver in the CBZ-treated SDT fatty rats: slight necrosis in the centrilobular hepatocytes, very slight to slight hypertrophy in the centrilobular hepatocytes, very slight to moderate fatty change in the hepatocytes and decreased glycogen deposition in the hepatocytes (Table 2). The light micrographs of the liver sections from the SDT fatty rats in the control and CBZ-treated groups were shown in Fig. 3. The incidence and severity of the treatment-related histopathological findings suggestive of hepatotoxicity were higher in the CBZ-treated SDT fatty rats than those in the CBZ-treated SD rats.

DISCUSSION

In the present study, we aimed to estimate the potential risk of CBZ-induced DILI in diabetic patients by using the SDT fatty rats as a pathological condition model for human T2DM.

Our previous investigation revealed that the characteristics of glucose metabolism (accelerated hepatic gluconeogenesis and fatty acid β-oxidation, decelerated glycolysis), liver function (higher plasma levels of AST, ALT, GGT and GLDH without liver injury) and hepatic glutathione synthesis (lower hepatic GSH and GSSG levels) in the SDT fatty rats are similar to those in T2DM patients (Takahashi *et al.*, 2019). In the present study, the control SDT fatty rats had higher body weights and food consumption (Fig. 1) and these were considered to be related to excessive eating of the diet due to introducing the *fa* allele of the Zucker fatty rat into the genome of this model rat (Shinohara *et al.*, 2000; Masuyama *et al.*, 2005). Hepatic function parameters in the present study, plasma AST and ALT levels were higher in the SDT fatty rats than in the SD rats and the higher plasma AST and ALT levels were not related to damage to the hepatocytes based on the results of histopathological examination. These findings in the plasma AST and ALT levels in the present study were considered to be related to acceleration of gluconeogenesis and protein catabolism in this animal model based on the results of our previous study (Takahashi *et al.*, 2019). The SDT fatty rats had slightly higher plasma GU levels and higher liver weights in the present study. The slightly higher plasma GU levels were not related to damage to the hepatocytes, as with the plasma AST and ALT levels and were considered to be related to the higher liver weights. The SDT fatty rats had higher hepatic LPO levels in the present study and the change were considered to be due to accelerat-

ed hepatic β-oxidation based on the results of our previous study (Takahashi *et al.*, 2019). The SDT fatty rats had lower hepatic GSH and GSSG levels than the SD rats in the present study and this was considered to be related to more preferential use of amino acids for gluconeogenesis rather than for glutathione synthesis based on the results of our previous study (Takahashi *et al.*, 2019).

CBZ was administered orally once daily to the SD and SDT fatty rats for 5 days and 3 or 4 days, respectively, at the dose levels of 0 (vehicle control) and 400 mg/kg (13 weeks of age at the initiation of dosing). There were no marked differences in the mortality between the CBZ-treated SD and SDT fatty rats. In the clinical observations, weakness of the muscle tone, abnormal gait, decreased locomotor activity and prone position were noted in all the CBZ-treated SD and SDT fatty rats within 1 hr after dosing from day 1. There were no differences in the intensity of these clinical signs (acute toxic response) between these two strains. Spread of seizure activity is suppressed by CBZ-treatment due to reduction in the post-tetanic potentiation of synaptic transmission (Clinical and Research Information on DILI by National Institutes of Health in the U.S.). In addition, the CBZ-treated rats had decreased locomotor and rearing activities (Nawakowska *et al.*, 2011). Thus, the clinical signs noted in the CBZ-treated SD and SDT fatty rats were considered to be related to the pharmacology of CBZ. The lack of differences in the intensity of the acute toxic response between the CBZ-treated SD and SDT fatty rats indicated that the systemic exposure to CBZ during the dosing period was similar between the CBZ-treated SD and SDT fatty rats.

In this study, it was demonstrated that hepatotoxicity was not noted in the histopathological examinations without any apparent increase in the plasma hepatic function parameters although plasma ALT levels were slightly increased in the CBZ-treated SD rats. The slightly increased plasma ALT levels in the CBZ-treated SD rats were considered to be related to the increased liver weights. These results were consistent with the results of the studies previously reported (Maheswari *et al.*, 2014; Iida *et al.*, 2015). On the other hand, in the CBZ-treated SDT fatty rats, slight necrosis was noted in the centrilobular hepatocytes and the plasma levels of ALT, GU and T-BIL were increased. In addition, the incidence and severity of fatty change in the periportal hepatocytes were increased in the liver in the CBZ-treated SDT fatty rats when compared with the CBZ-treated SD rats. Fatty change of the hepatocytes (steatosis) in non-clinical toxicity studies of drug candidates can be regarded as a critical finding for the estimation of their potential risk to induce DILI in humans when the fatty change is induced by mitochondrial dysfunction (Goda *et al.*, 2017). The mitochondrial dysfunction may have occurred in the hepatocytes in the CBZ-treated SDT fatty rats. Thus, the fatty change in the hepatocytes in the CBZ-treated SDT fatty rats was considered to be due to toxicity. From these, the CBZ-induced hepatotoxicity was detected only in the SDT fatty rats and was not detected in the SD rats under the conditions of this study.

Clinically apparent hepatotoxicity from CBZ-treatment is well described and the most common pattern of enzyme elevations in CBZ related DRESS syndrome is that of a mixed or cholestatic injury (the Clinical and Research Information on DILI by NIH in the U.S.). Liver biopsy shows the cholestatic injury with focal hepatocellular necrosis, prominence of eosinophils and occasionally granulomas (the Clinical and Research Information on DILI by NIH in the U.S.). In the present study, there were no treatment-related histopathological findings suggestive of cholestatic liver injury in the CBZ-treated SDT fatty rats. However, a marked increase in the plasma T-BIL levels were noted in one animal in the CBZ-treated SDT fatty rats and this change was considered to be related to the effects of CBZ on the hepatobiliary system in the SDT fatty rats. CBZ-related DRESS syndrome is one of the drug-induced hypersensitivity reactions (DHRs) and it has been reported that CBZ also induces Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (Schrijvers *et al.*, 2015). These adverse reactions reported in humans are considered to be immune mediated reactions. Useful animal models of DHRs are not available because DHRs cannot be detected in animals (Uetrecht, 2006) and the hepatotoxicity detected in the CBZ-treated SDT fatty rats in the present study was considered to be due to the direct effects (non-immune mediated cytotoxicity) of CBZ or its metabolites on the hepatocytes.

The development of CBZ-induced hepatotoxicity is somehow related to disturbance of glutathione metabolism (Kalapos, 2002). CBZ is metabolized by the hepatic CYP3A family. The main metabolic pathways of CBZ are epoxidation and hydroxylation, though conjugation reactions may also have a role (Maheswari *et al.*, 2014; Bernus *et al.*, 1996). 10,11-CBZ epoxide is the most important metabolic product of CBZ (Driefus and Langer, 1987). In the SDT fatty rats, the control levels of hepatic GSH and GSSG were lower than those in the SD rats and hepatic GSH and GSSG levels were decreased after treatment with CBZ. These indicate that detoxification capacity of 10,11-CBZ epoxide is lower in the SDT fatty rats than in the SD rats. Taking all the results of the present study into consideration, the enhancement of CBZ-in-

duced hepatotoxicity in the SDT fatty rats were considered to be due to insufficient hepatic glutathione levels for detoxification of the metabolic product of CBZ. Similar hepatotoxicity was also noted in the glutathione-depleted F344 rats by BSO after 5-day oral repeated dosing of CBZ at 400 to 600 mg/kg/day (Iida *et al.*, 2015) and the evidence strongly supports our discussion.

We have reported that the allyl alcohol induced hepatotoxicity is more prominent in the SDT fatty rats than in the SD rats (Takahashi *et al.*, 2019). Thus, we confirmed the usefulness of the SDT fatty rats for estimation of potential risk of chemical-induced hepatotoxicity in diabetic patients using two chemicals (allyl alcohol and CBZ). From these evidences, the potential risk of hepatotoxicity induced by chemicals which are detoxified via glutathione-conjugation are considered to be increased in the diabetic state due to depleted hepatic glutathione synthesis.

In conclusion, the CBZ induced hepatotoxicity in the SDT fatty rats, but not in the SD rats. This was considered to be related to lower hepatic detoxification capacity of the reactive metabolite of CBZ in the diabetic state. The results of the present study indicate that the potential risk of CBZ to induce DILI is higher in diabetic patients than in non-diabetic humans.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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