Prevention of Cadmium-Induced Testicular Toxicity by Oral Administration of Disulfiram in Rats

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The early testicular damage after acute cadmium (Cd) exposure and the prevention of Cd-induced testicular toxicity by the oral administration of disulfiram (DSF) at various times after Cd exposure in rats were studied. Although moderate enlargement in interstitial tissues was observed at the first 3 h after Cd injection, no morphological changes in the inside of seminiferous tubules were observed. At 6 h after Cd exposure, damage of both interstitial tissues and seminiferous tubules was observed. Rats received an intraperitoneal injection of DSF (0.5 mmol/kg) or an oral administration of DSF (0.5, 1.0 or 2.0 mmol/kg) 0.5, 1, 3 or 6 h after the subcutaneous injection of CdCl₂ (26.7 μ mol (3 mg) Cd/kg). The oral treatment with DSF at dose levels of 0.5 mmol/kg (0.5 or 1 h), 1.0 mmol/kg (3 h), or 2.0 mmol/kg (3 h) prevented the increase in testicular lipid peroxidation and calcium and Cd concentrations and the decrease in testicular weight, which were observed at 7 d after Cd injection, as well as by the injection of DSF at 0.5 mmol/kg (0.5 h). The results at 59 d after Cd injection indicated that the testicular Cd concentrations and testicular weight after DSF treatment were changed in sterile rats, but not in fertile animals. These results indicate that Cd-induced testicular toxicity occurs 3–6 h after Cd injection, and that more effective chelation therapy with DSF for testicular toxicity by Cd is considered to arise from the oral administration of DSF at dose levels of more than 1.0 mmol/kg within 3 h after acute Cd exposure in rats.

Key words — cadmium ; testicular toxicity ; chelate effect ; disulfiram

Introduction

Cadmium (Cd) has been shown to be toxic to numerous organs such as the liver, kidney, and testes.^{1–4)} Among these organs, the testes are extremely susceptible to Cd after parenteral exposure.⁵⁾ The deleterious effects of Cd on testicular function have been reported by several investigators.⁶⁾ The main symptoms of Cd-induced testicular toxicity include severe interstitial edema, followed by hemorrhage and necrosis with destruction of the seminiferous tubules.^{7–11)} However, the

sensitivity to Cd varies considerably depending on the dose, duration of treatment, or route of administration.^{12,13)} Chelation therapy for Cd has been effective in preventing the toxicity caused by Cd. We reported that diethyldithiocarbamate (DED) at 30 min after Cd injection was more effective than other dithiocarbamates in protecting against Cd-induced testicular toxicity.¹⁴⁾ Gale *et al.* reported that DED and disulfiram (DSF) had similar effects on the tissue distribution of Cd after the administration of Cd to mice.¹⁵⁾ DSF has been used in avoidance therapy for alco-

hol abuse since the late 1940's.¹⁶⁾ It has been reported that DSF is rapidly reduced to DED by the glutathione reductase system in erythrocytes.¹⁷⁾ Thus, DSF could conceivably exert the same effects on Cd toxicity as DED does.

The present study was carried out to examine early testicular toxicity after acute Cd exposure and the prevention of Cd-induced testicular toxicity by the oral administration of DSF at various time intervals after Cd exposure in order to evaluate the actual therapeutic effect of DSF in rats.

Materials and Methods

Materials — CdCl₂ and DSF were obtained from Wako Pure Chemical Ind. (Osaka). Other chemicals were of reagent grade.

Animals and Treatments — Male Kud: Wistar rats (SPF), weighing 180–220 g, were purchased from Kyudo Co., Ltd. (Kumamoto), and housed in individual metabolic cages with a commercial chow (Nosan Lab Chow) and drinking water *ad libitum* in temperature- and humidity-controlled rooms (12-h light/dark cycle). The rats were injected subcutaneously with saline (0.5 ml/animal) or CdCl₂ (26.7 μ mol (3 mg) Cd/kg) in 0.5 ml saline. At 0.5, 1, 3 or 6 h after Cd injection, they received an intraperitoneal (i.p.) injection of DSF (0.5 mmol/kg) in 0.5 ml or an oral administration of DSF (0.5, 1.0, or 2.0 mmol/kg) in 2 ml olive oil. Seven days after Cd administration the rats were killed by cervical fracture and the testes were removed. The testes were analyzed for lipid peroxidation. In the experiment for the determination of Cd, calcium (Ca) and iron (Fe), the rats received an i.p. injection of DSF at 0.5 h or an oral administration of DSF at 0.5, 1, 3 or 6 h after the s.c. injection of CdCl₂ (26.7 μ mol Cd/kg), and the testes were removed at 7 d after Cd injection. In the fertility test, male rats were killed after mating with female rats 59 d after Cd) and the testes were removed. Testes Cd concentrations were determined.

Lipid Peroxidation — Testes were homogenized with 9 volume of ice-cold 0.01 M

phosphate buffer (pH 7.4) containing 1.15% KCl and 0.025% ethylenediaminetetraacetic acid. Lipid peroxidation was assayed according to the thiobarbituric acid (TBA) method of Uchiyama and Mihara¹⁸⁾ using 1,1,3,3-tetraethoxypropane as the standard and expressed as nanomoles of TBA reactive substances (TBARS) per mg protein.

Protein — Protein was determined according to the method of Lowry *et al.*,¹⁹⁾ using bovine serum albumin as the standard.

Metals — Various organs were wet-ashed by the HClO₄-HNO₃ method, and metals (Cd, Ca and Fe) were determined using a Hitachi Z-8000 atomic absorption instrument.

Fertility Test — The fertility of male rats given Cd with and without a chelating agent was determined according to the method of Jackson *et al.*²⁰⁾ Each male rat was allowed to mate with a virgin female Kud: Wistar rat (SPF), weighing 230–240 g, which was purchased from Kyudo Co., Ltd. (Kumamoto), for 8 d, starting 52 d after the administration of Cd with and without a chelating agent. The fertility rate was expressed as the number of females impregnated *versus* the total number of males tested.

Histopathology — At 3, 6, and 24 h after the injection of Cd, male rats were lightly anesthetized with chloroform, and their testes were fixed with Bouin's solution by perfusion through the cannulated testicular artery. Then, the fixed testes were removed and placed in the same fixative overnight at 4°C with gentle agitation. After dehydration with a graded series of ethanol, the samples were embedded in paraffin, and then 4 μ m-thick sections were prepared. The sections were mounted on glass slides and then stained with hematoxylin and eosin for light microscopic examination.

Statistical Analysis — Data were analyzed by a one-way analysis of variance. When the analysis indicated that a significant difference existed, the treatment groups were compared to the controls by Duncan's new multiple range test. Probability values of less than 0.05 were considered to indicate significant differences.

Results

At the light microscope level, morphological changes of the testes were traced at 3, 6 and 24 h after Cd injection. When compared to the control (Fig. 1-A), no significant changes were observed at 3 h after Cd injection (Fig. 1-B) except for moderate enlargement of the interstitial tissue. As the exposure time increased, the lesion became more severe. At 6 h after Cd injection, the capillary lumen was likely enlarged, matrices were seen as edematous, and many fibrous structures were found (Fig. 1-C). At this time, the intercellular spaces between spermatogenic cells and Sertoli cells appeared to be enlarged. At 24 h after Cd exposure, inter-

cellular matrices and fibrous structures were extensively formed, while capillary lumens were narrowed (Fig. 1-D). In seminiferous tubules, germ cells were apparently sloughed from basal compartment. The sloughed cells showed pyknosis and karyorrhexis. The size of the seminiferous tubules diminished. The cell damage was irreversible, indicating cell death.

Lipid peroxidation in the testes of rats after the administration of Cd or Cd and DSF at various doses is shown in Table I. Cd administration significantly increased the lipid peroxidation in the testes. Oral administration of DSF (0.5 mmol/kg) at 0.5 or 1 h after Cd injection significantly prevented the increase in lipid peroxidation in the testes

Fig. 1. Light Micrograph of Rat Testes after Subcutaneous Injection of CdCl₂

(A) : Control. (B) : 3 h after injection. Interstitial tissues are moderately enlarged. (C) : 6 h after injection. Capillary lumen (arrow) is likely enlarged. Matrices are edematous, and many fibrous structures are seen (*). In seminiferous tubules, intercellular spaces between spermatogenic cells and Sertoli cells appear to be enlarged. (D) : 24 h after injection. In intercellular matrices, fibrous structures are extensively formed, while capillary lumen became narrowed (arrow). In seminiferous tubules, germ cells are sloughed from the basal compartment (*). The sloughed cells show pyknosis and karyorrhexis. Hematoxylin and eosin staining. $\times 50$.

TABLE I. Effect of DSF on Lipid Peroxidation in Testes of Rats after Cd Administration

Treatment	Dose of chelating agent (mmol/kg)	Lipid peroxidation ^{a)} (nmol TBARS/mg protein)
Control	—	0.43±0.02
Cd	—	0.89±0.03 ^{b)}
Cd+DSF (0.5 h, i.p.)	0.5	0.50±0.07 ^{c)}
Cd+DSF (0.5 h, p.o.)	0.5	0.45±0.06 ^{c)}
Cd+DSF (1 h, p.o.)	0.5	0.49±0.05 ^{c)}
Cd+DSF (3 h, p.o.)	0.5	0.54±0.02 ^{b,c)}
Cd+DSF (3 h, p.o.)	1.0	0.45±0.03 ^{c)}
Cd+DSF (6 h, p.o.)	1.0	0.58±0.05 ^{b,c)}
Cd+DSF (3 h, p.o.)	2.0	0.44±0.01 ^{c)}

Male, rats received the intraperitoneal or oral administration of DSF after the subcutaneous injection of CdCl₂ (26.7 μmol Cd/kg). a) The values represent the mean±S.D. for four animals. b) Significantly different from control, *p*<0.05. c) Significantly different from Cd, *p*<0.05.

within 7 d after Cd injection, as did DSF (0.5 mmol/kg) injection at 0.5 h after Cd. DSF (0.5 mmol/kg) administration at 3 h after Cd injection significantly prevented the increase in lipid peroxidation, but did not return it to the control level. DSF (1.0 mmol/kg) administration at 6 h after Cd injection significantly prevented the increase in lipid peroxidation, but did not return it to the control value.

A change in the testicular weight, expressed as a percentage of body weight, provides an additional indication of testicular toxicity.

As shown in Table II, Cd caused a significant decline in testicular weight, which decreased about 40% by 7 d. The decrease in testicular weight induced by Cd injection was completely prevented by the oral administration of DSF at doses of 0.5 mmol/kg (0.5 h) or 1.0 mmol/kg (3 h) or 2.0 mmol/kg (3 h) as well as by DSF injection at 0.5 mmol/kg (0.5 h). However, the decrease in testicular weight was not significantly prevented by the oral administration of DSF at doses of 0.5 mmol/kg (1 or 3 h after Cd injection) or 1.0 mmol/kg (6 h).

The tissue concentrations of Cd in rats treated with DSF after Cd are shown in Table III. The oral administration of DSF at doses of 0.5 mmol/kg (0.5 or 1 h), 1.0 mmol/kg (3 h) or 2.0 mmol/kg (3 h) significantly decreased the Cd concentrations in the testes at 7 d after Cd injection, as well as by DSF injection at 0.5 mmol/kg (0.5 h). DSF did not change the Cd concentration in the brain.

Table IV shows the concentrations of Ca and Fe in the testes after treatment with Cd with and without DSF. The Ca concentration in the testes significantly increased within 7 d after Cd injection, and the increase was significantly prevented by oral administration of DSF at doses of 0.5 mmol/kg (0.5 or 1 h), 1.0 mmol/kg (3 h) or 2.0 mmol/kg (3 h), as well

TABLE II. Effect of DSF on Testicular Weight of Rats after Cd Administration

Treatment	Dose of chelating agent (mmol/kg)	Testicular weight ^{a)} (% of body weight)		
		7 d	59 d	
			Fertile rats	Sterile rats
Control	—	1.04±0.06	0.85±0.04	—
Cd	—	0.61±0.03 ^{b)}	—	0.13±0.03 ^{d)}
Cd+DSF (0.5 h, i.p.)	0.5	1.09±0.05 ^{c)}	0.73±0.09	—
Cd+DSF (0.5 h, p.o.)	0.5	1.12±0.02 ^{c)}	0.75±0.13	0.35±0.04 ^{d)}
Cd+DSF (1 h, p.o.)	0.5	0.92±0.25	—	—
Cd+DSF (3 h, p.o.)	0.5	0.56±0.05 ^{b)}	0.76±0.05	0.21±0.04 ^{d)}
Cd+DSF (3 h, p.o.)	1.0	1.13±0.12 ^{c)}	0.81±0.07	—
Cd+DSF (6 h, p.o.)	1.0	0.62±0.06 ^{b)}	0.64±0.11	0.28±0.05 ^{d)}
Cd+DSF (3 h, p.o.)	2.0	1.12±0.17 ^{c)}	0.77±0.03	—

Male rats received the intraperitoneal or oral administration of DSF after subcutaneous injection of CdCl₂ (26.7 μmol Cd/kg). a) The values represent the mean±S.D. for four animals. b) Significantly different from control, *p*<0.05. c) Significantly different from Cd, *p*<0.05. d) Significantly different from control (fertility), *p*<0.05.

TABLE III. Effect of DSF on Cd Concentration in Testes and Brain

Treatment	Dose of chelating agent (mmol/kg)	Concentration of Cd ($\mu\text{g/g}$ wet tissue) ^{a)}			
		Testes			Brain
		7 d	59 d		7 d
			Fertile rats	Sterile rats	
Cd	—	0.94 ± 0.14	—	0.87 ± 0.01	0.20 ± 0.09
Cd+DSF (0.5 h, i.p.)	0.5	$0.44 \pm 0.11^b)$	$0.50 \pm 0.05^c)$	—	0.31 ± 0.03
Cd+DSF (0.5 h, p.o.)	0.5	$0.37 \pm 0.12^b)$	$0.37 \pm 0.08^c)$	0.81 ± 0.18	0.19 ± 0.02
Cd+DSF (1 h, p.o.)	0.5	$0.34 \pm 0.05^b)$	—	—	—
Cd+DSF (3 h, p.o.)	0.5	0.64 ± 0.24	$0.28 \pm 0.03^c)$	$0.64 \pm 0.05^c)$	—
Cd+DSF (3 h, p.o.)	1.0	$0.45 \pm 0.03^b)$	$0.31 \pm 0.04^c)$	—	0.16 ± 0.01
Cd+DSF (6 h, p.o.)	1.0	0.67 ± 0.16	$0.44 \pm 0.04^c)$	0.88 ± 0.05	—
Cd+DSF (3 h, p.o.)	2.0	$0.41 \pm 0.07^b)$	$0.32 \pm 0.07^c)$	—	0.19 ± 0.03

Rats received the intraperitoneal or oral administration of DSF after the subcutaneous injection of CdCl_2 ($26.7 \mu\text{mol Cd/kg}$). The rats were killed 7 d or 59 d after Cd administration, and Cd concentration was determined. a) The values represent the mean $\pm$ S.D. for four animals. b) Significantly different from Cd, $p < 0.05$. c) Significantly different from Cd (sterility), $p < 0.05$.

TABLE IV. Effect of DSF on Testis Concentration of Ca and Fe

Treatment	Dose of chelating agent (mmol/kg)	Concentration of metals ($\mu\text{g/g}$ wet tissue) ^{a)}	
		Ca	Fe
Control	—	23.3 ± 2.8	15.7 ± 3.0
Cd	—	$1396.5 \pm 28.9^b)$	$33.1 \pm 3.8^b)$
Cd+DSF (0.5 h, i.p.)	0.5	$20.4 \pm 2.2^c)$	$17.8 \pm 2.0^c)$
Cd+DSF (0.5 h, p.o.)	0.5	$24.0 \pm 1.5^c)$	$19.3 \pm 2.0^c)$
Cd+DSF (1 h, p.o.)	0.5	$26.9 \pm 2.8^c)$	$24.1 \pm 1.4^{b,c)}$
Cd+DSF (3 h, p.o.)	0.5	$241.7 \pm 92.0^{b,c)}$	$33.2 \pm 2.8^b)$
Cd+DSF (3 h, p.o.)	1.0	$24.1 \pm 1.4^c)$	$23.4 \pm 2.2^{b,c)}$
Cd+DSF (6 h, p.o.)	1.0	$2460.6 \pm 513.4^{b,c)}$	$30.1 \pm 8.9^b)$
Cd+DSF (3 h, p.o.)	2.0	$21.8 \pm 4.2^c)$	$20.6 \pm 1.0^{b,c)}$

Rats received the intraperitoneal or oral administration of DSF after the subcutaneous injection of CdCl_2 ($26.7 \mu\text{mol Cd/kg}$). a) The values represent the mean $\pm$ S.D. for four animals. b) Significantly different from control, $p < 0.05$. c) Significantly different from Cd, $p < 0.05$.

as by DSF injection at 0.5 mmol/kg (0.5 h). Cd caused a significant increase in the testicular Fe concentration, suggesting hemorrhage. The increase in the Fe concentration was completely prevented only by treatment with an oral administration of DSF at 0.5 mmol/kg (0.5 h) or by the injection of DSF at 0.5 mmol/kg (0.5 h), and returned to the control value. The increase in the Fe concentration was not prevented to the control level by treatment with DSF under other conditions.

The fertility of male rats treated with Cd

with and without DSF was also examined (Table V). Control male rats were fertile. None of the male rats given Cd alone was fertile. The sterility produced by Cd was almost completely prevented by treatment with an oral administration of DSF at 1.0 mmol/kg (0.5 or 3 h) or 2.0 mmol/kg (0.5 or 3 h), as well as by DSF injection at 0.5 mmol/kg (0.5 h). Treatment with an oral administration of DSF at 0.5 mmol/kg (0.5 h) or 1.0 mmol/kg (6 h) did not completely prevent the Cd-induced loss of fertility.

The concentrations of Cd in the testes

TABLE V. Effect of DSF on Fertility of Rats after Cd Administration

Treatment	Dose of chelating agent (mmol/kg)	Fertility rate
Control	—	7/7
Cd	—	0/7
Cd+DSF (0.5 h, i.p.)	0.5	7/7
Cd+DSF (0.5 h, p.o.)	0.5	5/7
Cd+DSF (3 h, p.o.)	0.5	3/7
Cd+DSF (0.5 h, p.o.)	1.0	7/7
Cd+DSF (3 h, p.o.)	1.0	7/7
Cd+DSF (6 h, p.o.)	1.0	3/7
Cd+DSF (0.5 h, p.o.)	2.0	7/7
Cd+DSF (3 h, p.o.)	2.0	7/7

Male rats received the intraperitoneal or oral administration of DSF after the subcutaneous injection of CdCl₂ (26.7 μ mol Cd/kg). The rat was mated with a virgin female rat for 8 d, starting 52 d after Cd. The fertility rate was expressed as the number of females impregnated *vs.* the number of males tested.

and the testicular weight of male rats mated with virgin female rats at 59 d after Cd administration were also measured. The decrease in testicular weight was prevented by treatment with DSF in fertile rats, but not by DSF in sterile rats (Table II). The concentrations of Cd in the testes was significantly decreased by treatment with DSF in fertile rats, but not by treatment with DSF in sterile rats (Table III).

Discussion

Our previous studies indicated that DED at dose levels of 1.0 and 3.0 mmol/kg protected against Cd-induced sterility.^{14,21)} The present study was carried out to examine the early testicular toxicity caused by Cd exposure and the prevention of Cd-induced testicular toxicity by the oral administration of DSF, which is metabolized to DED, at various times after Cd exposure in rats. It has been reported that Cd can result in the enhancement of lipid peroxidation in the testes^{22–24)} and in severe hemorrhagic necrosis of the testes.^{25,26)} Lipid peroxidation is an early and sensitive consequence of Cd exposure in

organs such as the testes, brain, liver and kidney.²⁷⁾ The present results show significant increases in lipid peroxidation and Cd, Ca and Fe concentrations in the testes, as well as a decrease in testicular weight, at 7 d after Cd exposure. The micrographical observation indicated an appearance of erythrocytes in the interstitial matrix at 12 h after Cd injection (data not shown). It has been reported that the hemoglobin concentrations in the testes of rats significantly increased after Cd injection and that the degree of hemorrhage in the testes was in good correlation with an increase in hemoglobin concentrations.²⁸⁾ The increase in the Fe concentration in the testes after Cd injection is considered to be attributed to hemorrhagic inflammation.^{14,21)} Thus, our results indicate that the increase in lipid peroxidation is related to the degree of hemorrhage, as reported by Sajiki *et al.*²³⁾ A marked increase in the testicular Ca concentration after Cd injection is similar to what has been reported in an earlier paper.^{14,21,29,30)} This is likely to result from cellular damage due to the Cd-induced lipid peroxidation in the testes. However, the detailed mechanisms by which the testicular Ca concentration increased after Cd injection should be further studied.

The histopathological results indicated that although enlargement and edema in the interstitial tissues were observed within the first 3 h after Cd injection, no morphological changes in the inside of the seminiferous tubules were observed compared to the control. At 6 h after Cd exposure, the damage in the side of seminiferous tubules was observed. Sertoli cells possess extensive tight junctions that form a physiological barrier between interstitial tissue and the interior of the seminiferous tubules. Therefore, it is possible that Cd-induced testicular toxicity was due to damage of inter-Sertoli cell tight junctions in the seminiferous tubules. The permeability barrier of the seminiferous epithelium was known to be affected by Cd before vascular

damage occurred.^{31,32)} Our findings support the hypothesis of Chubb³³⁾ that the progression of acute toxicologic actions of Cd begins with some damage of endothelial cells, which presumably increases vacuolization, and then vascular permeability increases, which leads to secondary damage of the seminiferous tubules.

Our previous study indicated that DED injection 0.5 h after Cd exposure protected against Cd-induced testicular toxicity.¹³⁾ To examine the actual therapeutic effect of DSF in Cd-induced testicular toxicity, DSF (0.5–2.0 mmol/kg) was administered orally at various time intervals after Cd exposure to rats. Cd-induced sterility was largely prevented by treatment with DSF (1.0 or 2.0 mmol/kg) at 3 h after Cd exposure, and such DSF treatment prevented the increase in lipid peroxidation and Cd and Ca concentrations and the decrease in testicular weight. Rats treated with DSF under other conditions experienced a less preventive effect on Cd-induced sterility. These groups prevented increases in lipid peroxidation and Cd, Ca and Fe concentrations and the decrease in testicular weight, all of which were observed at 7 d after Cd injection, but did not prevent them to the extent of maintaining control levels. They also consisted of the two groups in which such changes in the testes were significantly prevented and not prevented by DSF treatment (data not shown). The results at 59 d after Cd injection

indicated that the testicular Cd concentration and testicular weight after DSF treatment were significantly changed in the sterile rats, but not in the fertile animals. Thus, much less preventive effects of DSF on Cd-induced testicular damage, which were observed at 7 and 59 d after Cd, are considered to be closely related to the sterility of rats. This is presumably based on differences in susceptibility to Cd and on the metabolic rate of DSF in rats, but should be further studied.

DSF is rapidly reduced to DED by the glutathione reductase system in erythrocytes.¹⁷⁾ Approximately 80% of ingested DSF is absorbed from the intestinal tract of rats.³⁴⁾ DSF treatment significantly decreased the Cd concentrations in the testes, similarly to DED,¹⁴⁾ and did not cause the redistribution of Cd to the brain. These findings indicate that the protection provided by DSF against Cd-induced testicular damage is based upon DED formed by the metabolism of DSF, which was relatively rapidly absorbed from the intestines.

In summary, the present study indicates that Cd-induced testicular damage occurs within 3–6 h after Cd injection, and that more effective chelation therapy with DSF for testicular toxicity by Cd is anticipated to arise from the oral administration of DSF at doses of more than 1.0 mmol/kg within 3 h after acute Cd exposure in rats.

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