Preventive Effect of Pretreatment with Zinc on Cadmium Nephrotoxicity in Rats—Evaluation by Changes of Urinary Enzyme Activities—

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Abstract: The preventive effect of pretreatment with zinc (Zn) on cadmium (Cd) nephrotoxicity in rats was evaluated by the measurement of enzyme activity in urine. The enzymes examined were γ -glutamyltranspeptidase (γ -GTP, EC 2.3.2.2), glycylproline-diaminopeptidase (GP-DAP, EC 3.4.14.4), alkaline phosphatase (Al-P, EC 3.1.3.1) and leucine aminopeptidase (LAP, EC 3.4.11.1).

Three groups of male Sprague-Dawley rats were used. The first group received a single subcutaneous injection of 5.0 mg/kg body weight (5 rats, Cd Group), the second received a dosage of 250 μ mol Zn/kg 24 hours prior to the same dosage of Cd (5 rats, Zn-Cd Group) and the third received the same dosage of Zn (5 rats, Zn Group).

On the 1st day after Cd injection, significant elevation of both γ -GTP and GP-DAP activities in urine were observed in Cd Group, while in Zn-Cd Group the two enzyme activities were not elevated significantly compared to the reference values. On the 2nd day, γ -GTP and GP-DAP activities of Cd Group were reduced comparing with the first increase and those of Zn-Cd Group showed first a significant increase. On the 3rd day and after, both enzyme activities were almost the same as the reference value. The increase of enzyme activities in urine was explained to be caused by the leakage from renal cells. In Zn Group, both enzyme activities were observed little change in the course of the experiment.

The excretion pattern of Al-P and LAP into urine differed from those of γ -GTP and GP-DAP in all groups.

No histopathological changes under optical microscopy were observed in the livers and the kidneys of all groups.

From these results, it appears that Zn Pretreatment showed a preventive effect on Cd nephrotoxicity in terms of enzyme activities in urine before any histopathological changes were seen.

Key words: Cadmium nephrotoxicity—Zinc pretreatment—Urinary enzyme activity— γ -glutamyltranspeptidase—Glycylproline-diaminopeptidase—Alkaline phosphatase—Leucine aminopeptidase—Histopathological changes

INTRODUCTION

Since Parizek¹⁾ reported the preventive effect of Zn against the testicular atrophy caused by Cd, interest has increased in the interaction of Cd and Zn in vivo. As one of the other facts on the interaction, Leber *et al.*²⁾ reported that the administration of Zn prior to Cd challenge caused a pronounced increase in LD₅₀ in mice.

It has been known that the absorbed Cd tended to be retained for a long period, particularly in the liver and kidney, and caused renal proximal tube injury. In the previous paper,³⁾ the authors reported that the activities of rat urinary enzyme, especially γ -GTP and GP-DAP which located in the brush border of proximal tube in the kidney, were elevated significantly by a single injection of 5.0 mg/kg body weight of Cd. The measurement of these enzyme activities in rat urine was found to be a sensitive and easy screening test for the detection of renal damage.

In this study, the preventive effect of pretreatment with Zn on Cd nephrotoxicity was investigated by measuring the enzyme activities in urine.

MATERIALS AND METHODS

Chemicals:

L- γ -glutamyl-p-nitroanilide, p-nitrophenyl phosphoric acid disodium salt and glycylglycine were obtained from Nakarai Chemical Co. Ltd. L-leucine-p-nitroanilide was purchased from Peptide Institute Protein Research Foundation, Osaka, Japan. Glycylproline-p-nitroanilide tosylate was kindly supplied by Fujizoki Pharmaceutical Co., Tokyo, Japan. All other chemicals were of highest quality available.

Animals:

Male Sprague-Dawley rats (7 weeks old) with average body weight of 219 ± 21 g were used for the experiments. The animals had free access of standard rat chow and tap water. For urine collection, they were placed in metabolic cages which allowed quantitative separation of urine and feces. All 24-h urine were collected at room temperature of 25°C and their volume were measured, then the urine samples were centrifuged for 10 min at 3000 rpm and the supernatant was used for the assay of enzymes activities. Before the administration of Cd or Zn, the animals were accustomed to metabolic cages for 5 days. Then, on the 3 following day, the 24-h urines were collected and analysed as individual urine references.

In the first experiment, the rats were divided into three groups as follows: the first group received a single subcutaneous injection of 5.0 mg Cd/kg body weight into the right waist region of the back (5 rats, Cd Group), the second was injected with 250 μ mol Zn/kg into the left waist region of the back 24 hours prior to the same dosage of Cd (5 rats, Zn-Cd Group), and the third received the same dosage of Zn (5 rats, Zn Grup). On the 6th day after Cd injection, the

animals were sacrificed under ether anesthesia by cardiac puncture. The blood, liver and kidney were removed immediately. Serum was obtained by centrifuge for 10 min at 3000 rpm for the assay of enzymes activities. The liver and kidneys were homogenized in a glass homogenizer with 8 to 10 passes of a teflon pestle in 4 vol of 0.01 M Tris-HCl buffer solution (pH 8.0) with 0.25 M sucrose. The homogenize was diluted a suitable concentration for the assay of enzymes activities.

In the second experiment, eighteen rats were divided into four groups as follows: five rats were used as the Cd Group, four rats as the Zn-Cd Group, four rats as the Zn Group and five rats as control received no treatment. On the 1st day after Cd injection, the animals were sacrificed by the same manner and used for the assay of enzymes activities. This supplement experiment was performed after the first one, because the result of the first indicated that the observation on the 1st day after Cd injection was important.

Histopathological studies:

The liver and kidney were removed as soon as possible after death and fixed in formalin. Histopathological observation was performed after staining with hematoxylin-eosin.

Enzyme assays:

γ -GTP activity in rat urine was determined by a modification of the method of Szasz⁽¹⁾ using L- γ -glutamyl-p-nitroanilide and glycylglycine as the substrate. The standard incubation mixture contained 8.4 mmoles of L- γ -glutamyl-p-nitroanilide, 105.0 mmoles of glycylglycine and 200.0 mmoles of Tris-HCl buffer (pH 8.5) in a total volume of 2.0 ml. After preincubation of the mixture for 5 min at 37°C, the reaction was started by adding 5.0 μ l of rat urine. The reaction was stopped after 30 min by adding 2.0 ml of 1.5 M acetic acid, and light absorbance at 410 nm was read. GP-DAP activity was assayed by the method of Nagatsu *et al.*⁽⁵⁾ using glycylproline-p-nitroanilide tcsylate as substrate. The reaction mixture contained 150 mmoles Tris-HCl buffer (pH 7.9), 60 mmoles glycylglycine and 1.63 mmoles of glycylproline-p-nitroanilide tosylate in a total volume of 1.0 ml. After preincubation of the mixture for 5 min at 37°C, the reaction was started by adding 40.0 μ l of rat urine. The reaction was then terminated by adding 2.0 ml of 1.8% acetic acid and optical density at 410 nm was measured. One enzyme unit of both γ -GTP and GP-DAP activity were expressed as μ mole p-nitroanilide released enzymatically per min per liter of urine. γ -GTP activity in kidney was measured by adding 5.0 μ l of 20 times diluted homogenate. GP-DAP activity assayed by adding 10 μ l of serum or 20 μ l of 20 times diluted homogenate of kidney or liver. Al-P activity was measured according to the method of Bessey *et al.*⁽⁶⁾ using p-nitrophenyl phosphoric acid disodium salt as substrate. LAP activity was determined according to the method of Shimasue *et al.*⁽⁷⁾ using L-leucine-p-nitroanilide as substrate. Liver and serum enzymes examined were

GP-DAP, Al-P and LAP except for γ -GTP, because of low activity of γ -GTP. On the kidney, above four enzymes activities were assayed. Creatinine in urine was determined by the method of Bonsnes *et al.*⁸⁾

RESULTS

Body weight change during the experiment are shown in Fig. 1. In Zn Group, body weight increased gradually throughout the experiment, but body weight in both Cd Group and Zn-Cd Group were depressed on 2nd and 3rd day after Cd injection, and thereafter it was nearly constant in the both groups.

Table 1 summarises the changes of urinary parameters during the experiment. Both urine volume and creatinine content in urine of all the treated groups showed no change compared to each reference value. The values of enzyme activity in urine were also expressed in this table.

Fig. 2 shows the change of γ -GTP activity in urine expressed as a percent to reference value. On the 1st day after Cd injection, γ -GTP activity of Cd Group was significantly elevated ($p < 0.001$), whereas in Zn-Cd Group it was not elevated on the same day in spite of the same dosage of Cd. On the 2nd day, γ -GTP activity of Cd Group was decreased but was still significantly higher than the reference value ($p < 0.001$), while that of Zn-Cd Group was elevated significantly for the first time ($p < 0.001$). Thereafter in both Cd Group and Zn-Cd Group, they were depressed under the reference value. In Zn Group, γ -GTP activity showed no change compared to the reference value but on the 5th day, it was elevated ($p < 0.05$).

Fig. 1. Change of average body weight
 × — × : Zn Group △ — △ : Zn-Cd Group
 ○ — ○ : Cd Group

Table 1. Change of urinary parameters

Parameters	Reference value	Group	Day					
			0	1st	2nd	3rd	4th	5th
Urine volume (ml)	8.6± 2.3	Cd	—	10.1± 3.4	8.0± 3.5	10.2± 6.3	9.3± 2.5	10.4± 2.8
		Zn—Cd	8.0± 1.4	8.7± 1.4	9.4± 2.3	7.6± 2.0	8.4± 1.2	11.2± 1.8
		Zn	5.9± 1.6	7.6± 1.6	8.7± 1.6	7.4± 1.3	7.2± 1.4	8.4± 1.8
Creatinine (mg/ml)	159 ± 33	Cd	—	125 ± 54	139 ± 52	149 ± 59	139 ± 40	141 ± 29
		Zn—Cd	175 ± 41	154 ± 18	133 ± 70	174 ± 31	134 ± 29	121 ± 18
		Zn	209 ± 17	173 ± 29	162 ± 34	207 ± 30	186 ± 16	158 ± 18
γ -GTP (U/g. creatinine)	549.5±175.4	Cd	—	1425.1±667.0	1028.1±244.8	444.4±113.5	427.9±218.3	352.1±112.6
		Zn—Cd	689.1±197.4	651.9±324.4	1105.9±543.5	476.6±159.4	440.8±119.7	406.4±159.3
		Zn	666.5±212.6	463.7± 64.2	517.3± 62.6	506.1± 65.6	670.6± 75.9	747.1±123.1
GP-DAP (U/g. creatinine)	118.9± 30.2	Cd	—	256.1±113.1	160.5± 26.7	90.1± 17.7	78.2± 15.2	62.4± 19.1
		Zn—Cd	159.9± 57.9	135.4± 39.8	178.8± 73.5	99.9± 19.9	114.5± 26.4	110.9± 31.5
		Zn	136.9± 37.2	113.0± 15.0	120.4± 10.4	110.2± 18.9	133.8± 14.1	148.1± 12.4
ALP (BL-U/g. creatinine)	137.6±107.7	Cd	—	52.9± 42.1	183.7± 95.9	89.4± 30.7	206.0±203.1	228.9± 98.3
		Zn—Cd	288.7±300.2	292.0±286.9	239.9±166.4	161.0± 82.6	130.1± 78.4	132.6± 54.7
		Zn	388.0±244.0	299.2±240.7	222.8± 31.5	168.5± 58.5	158.9±103.0	112.5± 46.9
LAP (U/g. creatinine)	33.5± 11.2	Cd	—	24.3± 8.8	19.0± 4.9	16.4± 2.8	20.7± 5.8	4.9± 3.1
		Zn—Cd	17.7± 6.4	16.5± 7.7	39.3± 26.9	11.5± 1.0	22.4± 3.3	10.7± 3.9
		Zn	17.2± 5.2	15.7± 2.3	32.4± 3.4	25.5± 4.4	33.3± 3.7	23.3± 4.7

Reference values were obtained by examining 24-h urine collected before Cd or Zn administration (n=50).

All data are expressed as mean value±S.D. (n=5).

Fig. 2. Variation of daily γ -GTP activity in urine

Results are expressed as a percent of reference value. Analysis against reference value of the data was carried out by using student's t-test.

a) $p < 0.001$; b) $p < 0.01$; c) $p < 0.05$

Fig. 3. Variation of daily GP-DAP activity in urine

Results are expressed as a percent of reference value. Symbols are the same as in Fig. 2.

Fig. 4. Variations of daily A1-P activity in urine
Results are expressed as a percent of
reference value. Symbols are the same
as in Fig. 2.

In all groups, the changing patterns of GP-DAP activity were very similar to those of γ -GTP activity (Fig. 3). On the 1st day after Cd injection, GP-DAP activity of Cd Group was also elevated significantly ($p < 0.001$), but in Zn-Cd Group it was not elevated. On the 2nd day, GP-DAP activity of Cd Group was still higher and that of Zn-Cd Group was also significantly elevated ($p < 0.01$). Thereafter GP-DAP activities of both groups were depressed under the reference value, especially in Cd Group the depression were significant on the 3rd ($p < 0.05$), the 4th ($p < 0.01$) and the 5th day ($p < 0.001$).

On 0 and the 1st day, A1-P activities of Zn-Cd Group and Zn Group were significantly elevated (Fig. 4). A1-P activity in rat urine had a large deviation value in all groups as indicated in Table 1. Therefore there were no significant changes in any group compared to the reference value.

LAP activities in all groups were depressed significantly under the reference value except for some of Zn-Cd Group and Zn Group (Fig. 5).

Table 2 shows enzyme activities in the liver on the 1st and the 6th day after Cd injection. GP-DAP activity in the liver of Cd Group on the 1st day was significantly depressed compared to the control value but that of Zn-Cd Group on the same day was not influenced in spite of the same dosage with Cd. A1-P

Fig. 5. Variations of daily LAP activity in urine

Results are expressed as a percent of reference value. Symbols are the same as in Fig. 2.

Table 2. Enzyme activity in the liver on the 1st and 6th day after Cd injection

	Group	GP-DAP U/g. tissue	Al-P BL-U/g. tissue	LAP U/g. tissue
1st day after Cd injection	Cd	9.60 ^{e)} ± 1.08	99.1 ^{b)} ± 37.8	2.59 ± 0.14
	Zn-Cd	10.31 ± 1.05	91.8 ^{b)} ± 29.7	2.94 ± 0.36
	Zn	11.51 ± 1.13	45.3 ^{b)} ± 8.4	2.72 ± 0.50
6th day after Cd injection	Cd	9.95 ± 1.78	73.2 ^{b)} ± 12.2	2.30 ± 0.38
	Zn-Cd	11.32 ± 1.81	94.6 ^{b)} ± 16.3	3.39 ± 0.91
	Cd	10.70 ± 2.03	32.8 ^{e)} ± 6.9	2.69 ± 0.54
	Control	11.71 ± 1.22	21.5 ± 5.3	2.59 ± 0.23

All data are expressed as mean value ± S.D. (n=5).

Analysis against control value of the data was carried out by using student's t-test.

^{b)} $p < 0.01$; ^{e)} $p < 0.05$

activities in the liver of all groups on the 1st and the 6th were elevated significantly compared to the control value. This phenomena seem to be difficult to interpret at the present stage and its requires further investigation. LAP activities in the liver of all groups on both days showed any significant changes compared to the control value.

Activities of the enzymes in serum on the 1st and 6th day after Cd injection were summarized in Table 3. GP-DAP activity of Cd Group on the 1st day showed a tendency to increase, but no significant difference was observed compared to the control value ($p < 0.20$). Al-P activities of Cd Group and Zn-Cd Group

Table 3. Enzyme activity in rat serum on the 1st and 6th day after Cd injection

	Group	GP-DAP mU/ml	Al-P BL-U/ml	LAP mU/ml
1st day after Cd injection	Cd	133.6±81.0	2.92 ^{b)} ±0.16	16.0 ^{b)} ±2.2
	Zn-Cd	61.3±18.3	3.77 ^{b)} ±0.23	15.2 ^{c)} ±6.9
	Zn	61.0± 4.2	5.19 ±0.13	16.2 ^{b)} ±3.3
6th day after Cd injection	Cd	66.4±12.4	3.61 ±1.56	24.5 ±2.1
	Zn-Cd	85.1±17.7	5.76 ±1.08	31.8 ^{b)} ±3.1
	Zn	63.6± 9.1	8.17 ^{a)} ±0.99	24.7 ±4.3
	Control	73.6± 7.4	4.96 ±0.88	25.1 ±2.2

All data are expressed as mean value±S.D. (n=5).

Analysis against control value of the data was carried out by using student's t-test.

a) p<0.001; b) p<0.01; c) p<0.05

Table 4. Enzyme activity in rat kidney on the 1st and 6th day after Cd injection

	Group	GP-DAP U/g. tissue	γ -GTP U/g. tissue	Al-P U/g. tissue	LAP U/g. tissue
1st day after Cd injection	Cd	64.89 ^{c)} ±7.03	413.5 ±28.2	1884.0 ^{c)} ±236.0	11.14 ±1.70
	Zn-Cd	78.54 ±3.25	486.6 ±31.0	2588.4 ±498.5	15.16 ±5.50
	Zn	74.85 ±1.89	443.3 ±28.5	2453.2 ±257.2	23.24 ±3.21
6th day after Cd injection	Cd	71.62 ±5.20	420.2 ±43.3	2222.9 ±132.9	6.41 ^{b)} ±2.36
	Zn-Cd	70.48 ±6.14	459.0 ±37.3	2075.2 ±254.4	10.54 ^{c)} ±4.67
	Zn	66.27 ^{c)} ±7.04	383.8 ^{b)} ±30.8	2199.6 ±176.6	15.24 ±6.39
	Control	78.12 ±6.90	467.5 ±45.4	2627.0 ±516.3	21.84 ±9.27

All data are expressed as mean value±S.D. (n=5).

Analysis against control value of the data was carried out by using student's t-test.

a) p<0.001; b) p<0.01; c) p<0.05

on the 1st day were decreased significantly, but that of Zn Group on the 6th day was significantly elevated. LAP activities of all groups on the 1st day were decreased, but on the 6th day they were recovered to the control value.

Table 4 shows enzyme activities in the kidneys on the 1st and 6th day after Cd injection. GP-DAP activities in the kidneys of Cd Group on the 1st day was decreased significantly, but on the same day that of Zn-Cd Group showed no change compared to the control value. On the 6th day, GP-DAP activity of Zn Group was depressed significantly. The changing pattern of γ -GTP activity in the kidney were very similar to those of GP-DAP activity. γ -GTP activity of Cd Group on the 1st day was slightly decreased (-11.6%; p<0.20), but on the same day that of Zn-Cd Group showed no change compared to the control value. Al-P activity of Cd Group on the 1st day was decreased significantly, and those of all groups on the 6th day was inclined to be lower than the control value but not significant. LAP activities of Cd Group on both the 1st and 6th day were depressed significantly, but such depression effect was not so large in Zn-Cd Group.

No histopathological changes under optical microscopy were observed in the

liver and the kidney of any groups except for slight congestion of glomeruli in Cd Group and Zn-Cd Group.

DISCUSSION

The measurement of the enzyme activity in urine has been used as a sensitive, nondestructive and easy screening test for the detection of renal damages caused by some nephrotoxins.⁹⁻¹³⁾ For example, Flora *et al.*¹⁴⁾ demonstrated that the protective effect of selenium against cadmium-induced nephrotoxicity and hepatotoxicity could be evaluated by measuring the activities of diagnostic enzymes in serum and urine. Sener *et al.*¹⁵⁾ also reported that the urinary excretion of γ -GTP as an indicator of renal damage caused by HgCl_2 was decreased by the simultaneous administration of equimolar dose of selenite when both compounds were given i.p. for several days. The authors showed previously the elevation of γ -GTP and GP-DAP activities in urine of rats injected with Cd. As described already, the toxicity of Cd was found to be reduced by preliminary administration of Zn as reported by Parizek¹⁾ and Leber *et al.*²⁾

Basing upon these results, the authors tried to evaluate the effect of pretreatment with Zn on Cd nephrotoxicity by the measurement of the enzyme activity in rat urine.

The injection of Cd alone induced significant elevation of the urinary enzyme activities (especially γ -GTP and GP-DAP) on the 1st day after the injection. The elevation of enzyme activities was found to be significantly reduced by the pretreatment with Zn 24 hours before Cd injection. There was significant positive correlation between γ -GTP activity and GP-DAP activity in urine ($r=0.863$, $n=135$). Because these two enzymes are known to be present in the brush border of proximal tubule in the kidney, it is probable that the elevation of γ -GTP and GP-DAP activity in rat urine are caused by the leakage from renal cells.

The urinary excretion patterns of LAP and Al-P differed from those of γ -GTP and GP-DAP in Cd, Zn-Cd and Zn Groups. Both Al-P and LAP are metallo-enzyme contained Zn in its molecule and Zn may be replaced by Cd, so it may be possible that their activity are reduced directly by Cd. But this assumption is difficult to confirm at present stage and requires further investigation.

Enzyme activities in rat serum and kidney as the possible sources of urinary enzymes were examined in the renal damage caused by Cd. γ -GTP activity in rat serum was too low to detect. There was no relationship between the serum and urinary GP-DAP activities ($r=0.070$, $n=29$). Accordingly, it was concluded that urinary γ -GTP and GP-DAP did not depend upon the serum enzymes. When both the activities of γ -GTP and GP-DAP elevated in rat urine on the 1st day after Cd injection, the activities in the kidney showed a tendency to decrease. Furthermore, the assay of γ -GTP activity in urine has been applied generally for the test of renal function. These facts indicate a close correlation between the

loss of kidney enzymes and the elevation of urinary enzymes in the renal damage by Cd. From the above results, finally it may be concluded that Cd causes the leakage of γ -GTP and GP-DAP from renal cells into urine before the appearance of any histopathological changes and Zn pretreatment 24 hours before Cd injection reduces the enzyme leakage.

In the present experiment, we could prove the preventive effect of pretreatment with Zn on Cd nephrotoxicity by the measurement of the enzyme activities in rat urine. However, the mechanism of this phenomenon remained obscure and further investigation is necessary.

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