Effects of chlorpromazine and verapamil on nephrotoxicity of cadmium in rats

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Abstract: **AIM** To study whether chlorpromazine (CPZ) and verapamil (Ver) have protective effects on the nephrotoxicity of cadmium (Cd). **METHODS** Thirty-two Wistar rats were divided randomly into four groups. Each agent was injected 5 times per week for 6 weeks. The rats in Cd-treated group were sc injected with $CdCl_2$ 7 $\mu mol \cdot kg^{-1}$. The rats of CPZ- and Ver- pretreated group were ip injected with CPZ 5 $mg \cdot kg^{-1}$, Ver 4 $mg \cdot kg^{-1}$, respectively, 1 h later sc injected with $CdCl_2$ 7 $\mu mol \cdot kg^{-1}$. The control group was sc injected with saline 2 $mL \cdot kg^{-1}$ at corresponding time. Twenty-four hours after the last injection, the 24-h urine samples were collected. The renal cortex was also excised. Lactate dehydrogenase (LDH) activity, protein and Cd concentration in urine were determined. The activities of protein kinase C (PKC), Na^+-K^+-ATPase, Ca^{2+}-ATPase and Cd concentration of renal cortex were also measured. **RESULTS** Cd concentrations of renal cortex and urine in rats from Cd-treated group were significantly higher than those of control group. Cd concentrations in urine of rats from CPZ- and Ver-pretreated groups were significantly lower than those of Cd-treated group, but there was no significant change in renal cortex. As compared with control group, LDH activity, protein content in urine and the activities of PKC, Na^+-K^+-ATPase and Ca^{2+}-ATPase in the rats of Cd-treated group increased significantly. LDH activity, protein content in urine and activities of PKC, Na^+-K^+-ATPase and Ca^{2+}-ATPase in rats of CPZ- and Ver-pretreated groups were significantly lower than those of Cd-treated group. **CONCLUSION** Cd could activate the activities of PKC, Na^+-K^+-ATPase and Ca^{2+}-ATPase. Moreover, pretreatment of CPZ and Ver could reduce nephrotoxicity of Cd.

Key words: cadmium; chlorpromazine; verapamil; nephrotoxicity

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Cadmium (Cd), a potent toxic metal, is very harmful to the environment and the humans being because of its accumulation in liver, kidney and other tissues. Chronic cadmium exposure causes renal proximal tubular cell injury. Cd affects cellular functions by perturbing signal transductions, such as protein kinase C (PKC), mitogen-activated protein kinase and cyclic AMP pathways, and interaction with the cellular calcium (Ca) metabolism[1]. Recent study demonstrated that Cd directly disturbs intracellular Ca^{2+} homeostasis which contributes an important aspect of Cd-resulted body injury[2]. Calmodulin (CaM) is a ubiquitous calcium-binding protein that regulates a wide variety of calcium-dependent enzymes and processes. Cd can replace Ca, inducing a similar conformational change in CaM and thus makes the CaM free from the control of Ca, which could disturb many cellular functions.

Chlorpromazine (CPZ) is the best known and most commonly used antipsychotic. It has been reported that CPZ can block sodium, calcium, and potassium channels[3] and one of the actions of CPZ is to inhibit CaM activity[4]. Previous study has shown that CPZ and other psychotropic drugs could reduce the adverse effect of Cd resulted from its anticalmodulin activity on testes[5]. Recent study has demonstrated that blockage of receptor operated Ca^{2+} channels in WRL-68 cells inhibited Cd uptake by as much as 51%[6]. Furthermore, the blockage of voltage-sensitive Ca^{2+} channels has been shown to inhibit Cd uptake and decrease

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Cd toxicity in an established secretory cell line[7]. These findings suggest that Cd uptake through Ca^{2+} transport pathways be an important biological process leading to Cd toxicity. Thus, the blockage of Ca^{2+} channels with verapamil (Ver) can significantly reduce Cd toxicity.

However, Lermioglu, *et al*[8] reported that, although interfering with the metabolism of Cd, CPZ and Ver did not prevent renal damage in rats chronically exposed to this heavy metal. We suspect that the single index CC16 they examined in their study is not enough to resplect the pathological features of renal damage. The objective of the present study is to examine whether Cd-induced nephrotoxicity is involved in the abnormal activation of PKC and ATPase, and, if so, whether CPZ and Ver can reduce the adverse effect of Cd-induced.

1　MATERIALS AND METHODS

1.1　Animals treatment

Wistar rats (150 ± 10)g $(\bar{x} \pm s)$ were obtained from the Experimental Animal Department of China Medical University. Thirty-two Wistar rats were divided randomly into 4 groups: each group containing 4 ♀ and 4 ♂, control group, Cd-treated group, and CPZ-, and Ver-pretreated groups. Cd-treated group rats were sc injected with $CdCl_2$ 7 mol·kg^{-1}. CPZ- and Ver-pretreated groups were ip injected with CPZ 5 mg·kg^{-1}, or Ver 4 mg·kg^{-1}, respectively, 1 h later sc injected with $CdCl_2$ 7 μmol·kg^{-1}. The control group was sc injected with saline 2 mL·kg^{-1} at corresponding time. All rats were injected 5 times per week for 6 weeks.

1.2　Sample collection and preparation

After the last injection, the rats were held in metabolic cages for 24 h urine samples. Lactate dehydrogenase (LDH) activity, protein level and Cd concentration in urine were analyzed in freshly collected samples from metabolic cages. Then, the rats were sacrificed by ether. The renal cortex samples were quickly excised, rinsed in ice-cold saline to clear them of blood, and 5% (*W*/*V*) homogenate was prepared with buffer A (containing 250 mmol·L^{-1} sucrose, 10 mmol·L^{-1} imidazole-HCl, pH 7.4) for assay of PKC, Na^+-K^+-ATPase and Ca^{2+}-ATPase activities.

1.3　Assay of kidney damage indicators

Urinary protein was determined by a dye-binding method[9], using bovine serum albumin as the standard. LDH activity was measured by the method of Hochella, *et al*[10]. Urinary creatinine (Cr) was measured by the method of Heinegard, *et al*[11]. Cd concentrations in renal cortex and urine were determined by atomic absorbance spectrophotometer. The indicators in urine were revised by urinary Cr. Cd concentration in renal cortex was revised by tissue weight.

1.4　Assay of PKC activity

The samples were separated by ultracentrifugation (100 000 × *g*) at 4℃ for 60 min. The supernatant (cytoplasm fraction) was kept at 4℃, whereas the pellet was resuspended in 1 mL buffer B containing 1% Triton X-100, 5 mmol·L^{-1} EDTA, 2 mmol·L^{-1} EGTA and kept at 4℃ for 12 h, then ultracentrifugation (100 000 × *g*) at 4℃ for 60 min. The supernatant was cytomembrane fraction[12]. PKC activities were determined as the incorporation of ^{32}P from [γ-^{32}P]ATP into histone Ⅲ-S[13]. The catalytic reaction was performed in a total volume of 100 μL containing 20 mmol·L^{-1} Tris-HCl pH 7.4, 10 mmol·L^{-1} $MgCl_2$, 0.5 mmol·L^{-1} $CaCl_2$, 4 μg diolein, 40 μg phosphatidylserine, 20 μg histone Ⅲ-S, 50 μmol·L^{-1}[γ-^{32}P] ATP (370 000 Bq·L^{-1}). The reaction was initiated by the addition of 20 μL sample, which was incubated for 10 min at 30℃ in the reaction mixture, and then immediately spotted onto filter paper (Xing-hua type Ⅲ). The filters were then washed two times with 5 mL of 5% trichloroacetic acid (TCA), dissolved in scintillation fluor, and counted for radioactivity. The radioactivity was measured by scintillation counter (Beckman). PKC activity is defined as the amount of [γ-^{32}P] ATP (nmol) being transferred to histone Ⅲ-S by the enzyme during 1 min by 1 g protein of the samples.

1.5　Assay of Na^+-K^+-ATPase and Ca^{2+}-ATPase activities

Tissue samples were centrifuged at 8000 × *g*

for 10 min. The supernatant was centrifuged at 10 000 × g for 10 min again. The pellet was resuspended in 1 mL buffer A for assay. ATPase activity was assayed by colorimetric method[14] and recorded as the amount of inorganic phosphate (Pi) released from ATP. The reaction medium 600 μL was contained 25 $mmol \cdot L^{-1}$ KCl, 120 $mmol \cdot L^{-1}$ NaCl, 4 $mmol \cdot L^{-1}$ $MgCl_2$, 4 $mmol \cdot L^{-1}$ Na_2ATP and 60 $mmol \cdot L^{-1}$ Tris-HCl buffer (pH 7.4 at 37℃). The reaction was initiated by addition of sample followed by incubation at 37℃ for 10 min and was stopped with TCA. The inorganic phosphate-molybdate complex was quantitated by spectrophotometer at 706 nm. Total ATPase activity was measured in the presence of Na^+, K^+ and Mg^{2+} ions, and Mg^{2+}-ATPase activity, with the ions and 5 $mmol \cdot L^{-1}$ ouabain. The Na^+-K^+-ATPase activity was calculated as the difference between the total ATPase activity and the Mg^{2+}-ATPase activity. In the presence of Ca^{2+}, Ca^{2+}-ATPase activity was appeared. Ca^{2+}-ATPase activity was measured in the presence of Ca^{2+}, Na^+, K^+, Mg^{2+} ions and 5 $mmol \cdot L^{-1}$ ouabain. The ATPase activity was expressed as mmol of liberated inorganic Pi by 1 g of protein per hour.

1.6 Statistical analysis

All data were presented $\bar{x} \pm s$. Statistical comparisons were performed based on analysis of variance (ANOVA) followed by Student-Newman-Keuls test at $P < 0.05$.

2 RESULTS

2.1 LDH activity and protein level in urine

The data in Tab 1 indicated that urinary LDH activity and urinary protein content in Cd-treated group were significantly higher than that of control group ($P < 0.01$). As compared with Cd-treated group, LDH activity and protein content in urine significantly decreased in CPZ-pretreated group ($P < 0.05$). Ver-pretreatment suppressed the rise in urinary LDH activity and urinary protein content but they were still higher than that of control group.

Tab 1. Effect of chlorpromazine (CPZ) and verapamil (Ver) on lactate dehydrogenase (LDH) activity and protein level in urine

Group	LDH/$\mu mol \cdot g^{-1} \cdot min^{-1}$	Protein/$g \cdot g^{-1}$ Cr
Control	1.3 ± 1.2	0.56 ± 0.08
Cd	13.4 ± 7.4**	2.16 ± 0.59**
CPZ + Cd	7.7 ± 2.7**#	1.72 ± 0.29**#
Ver + Cd	9.1 ± 2.4**	1.65 ± 0.13**#

The control group received sc injections of normal saline 2 $mL \cdot kg^{-1}$; the Cd-treated group received sc injections of $CdCl_2$ 7 $\mu mol \cdot kg^{-1}$; the CPZ- and Ver-pretreated groups were ip injected with 5 $mg \cdot kg^{-1}$ CPZ and 4 $mg \cdot kg^{-1}$ Ver, respectively, then with sc injection of $CdCl_2$ 7 $\mu mol \cdot kg^{-1}$ 1 h later; all agents injected 5 times per week for 6 weeks. Cr: creatinine. $\bar{x} \pm s$, $n = 8$. ** $P < 0.01$, compared with control group; # $P < 0.05$, compared with Cd-treated group.

2.2 Cadmium concentrations in renal cortex and urine

The data in Tab 2 indicated that Cd concentrations in renal cortex and urine of Cd-treated group were significantly higher than that of control group ($P < 0.01$). As compared with Cd-treated group, urinary Cd concentrations significantly decreased in CPZ-, or Ver-pretreated groups. However, Cd concentration in renal cortex was not significantly different between Cd-treated group and CPZ-, or Ver-pretreated groups.

Tab 2. Effect of CPZ and Ver on cadmium concentrations in renal cortex and urine

Group	Renal cortex Cd/ $\mu g \cdot g^{-1}$ tissue	Urine Cd/ $mg \cdot g^{-1}$ Cr
Control	0.25 ± 0.22	0.031 ± 0.026
Cd	145.8 ± 13.3**	1.18 ± 0.54**
CPZ + Cd	148.9 ± 18.9**	0.26 ± 0.21**##
Ver + Cd	132.5 ± 15.9**	0.74 ± 0.36**#△

See Tab 1 for the treatment. $\bar{x} \pm s$, $n = 8$. ** $P < 0.01$, compared with control group; # $P < 0.05$, ## $P < 0.01$, compared with Cd-treated group; △ $P < 0.05$, compared with CPZ + Cd group.

2.3 PKC activity in renal cortex

The data in Tab 3 indicated that PKC activities in cell membrane and cytoplasm of renal cortex of Cd-treated group were significantly higher than that of control group ($P < 0.01$). As compared with control group, PKC activity in cell

plasm of renal cortex significantly decreased in CPZ-pretreated group ($P < 0.01$). As compared with Cd-treated group, both PKC activities in cell membrane and cytoplasm decreased to some extent in CPZ-pretreated group. PKC activity in cell membrane of Ver-pretreated group was significantly lower than that of Cd-treated group ($P < 0.05$). As compared with Ver-pretreated group, PKC activity in cytoplasm of CPZ-pretreated group significantly decreased ($P < 0.01$).

Tab 3. Effect of CPZ and Ver on protein kinase C (PKC) activity in renal cortex

Group	PKC/nmol·g^{-1}·min^{-1}	
	Cell membrane	Cytoplasm
Control	36 ± 4	36 ± 7
Cd	51 ± 9**	43 ± 7*
CPZ + Cd	41 ± 5$^{\#}$	27 ± 5$^{**\#\#}$
Ver + Cd	42 ± 5$^{\#}$	39 ± 4$^{\triangle\triangle}$

See Tab 1 for the treatment. $\bar{x} \pm s$, $n = 8$. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with Cd-treated group; $^{\triangle\triangle}P < 0.01$, compared with CPZ + Cd group.

2.4 Na^+-K^+-ATPase and Ca^{2+}-ATPase activities in renal cortex

The data in Tab 4 indicated that Na^+-K^+-ATPase and Ca^{2+}-ATPase activities in renal cortex of Cd-treated group were significantly higher than that of control group ($P < 0.01$). As compared with Cd-treated group, Ca^{2+}-ATPase activity significantly decreased in CPZ-pretreated group ($P < 0.01$), but Na^+-K^+-ATPase activity did not significantly change. Na^+-K^+-ATPase and Ca^{2+}-ATPase activities were not only lower than that of Cd-treated group ($P < 0.01$) but also were lower than that of CPZ-pretreated group ($P < 0.05$).

Tab 4. Effect of CPZ and Ver on activities of Na^+-K^+-ATPase and Ca^{2+}-ATPase in renal cortex

Group	Na^+-K^+-ATPase/ mmol·g^{-1}·h^{-1}	Ca^{2+}-ATPase/ mmol·g^{-1}·h^{-1}
Control	3.3 ± 0.8	1.5 ± 0.3
Cd	8.3 ± 1.4**	3.4 ± 0.8**
CPZ + Cd	7.4 ± 1.6**	2.5 ± 0.7$^{**\#}$
Ver + Cd	5.8 ± 1.2$^{**\#\#\triangle}$	1.7 ± 0.5$^{\#\#\triangle}$

See Tab 1 for the treatment. $\bar{x} \pm s$, $n = 8$. $^{**}P < 0.01$, compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with Cd-treated group; $^{\triangle}P < 0.05$, compared with CPZ + Cd group.

3 DISCUSSION

That urinary protein and LDH activity are related to Cd-induced nephrotoxicity has been shown previously[15]. Thus, it is rational to assume that changes in urinary protein and LDH activity in the present study are due to the toxic effects of Cd exposure on the renal proximal tubular epithelium. Our present experiment showed that LDH activity in Cd-treated group was 9.3-fold greater than control value and the urinary protein and Cd concentrations followed a trend similar to LDH.

Ca^{2+} extrusion from the cells by Ca^{2+}-ATPase and Na^+-K^+-ATPase is of crucial importance for maintaining a low resting intracellular Ca^{2+} concentration[16]. It has been reported that long-term Cd administration (1 mg·kg^{-1} rat daily for 4 months) activated brain acetyl cholinesterase (AChE) and Na^+-K^+-ATPase about 50% – 65% ($P < 0.001$) but not Mg^{2+}-ATPase[17]. PKC is considered integral to signal transduction *via* the Ca messenger system. In this study, Cd causes an activation of PKC and increase in Na^+-K^+-ATPase and Ca^{2+}-ATPase activities. It may be because Cd^{2+} disturbs intracellular Ca homeostasis which results in intracellular Ca^{2+} overloading. Intracellular Ca^{2+} or Cd^{2+} can activate CaM, at the same time, and stimulate CaM-dependent enzymes, such as Na^+-K^+-ATPase and Ca^{2+}-ATPase and so on. Another possible mechanism is that Cd^{2+} activates PKC and subsequently increases Ca^{2+} cycling across the plasma membrane[18].

Recent reports indicated that CaM inhibitors, such as CPZ, pretreatment has an effect on Cd-induced testicular toxicity in rats with modifying Cd distribution[19]. In the present experiment, CPZ-pretreatment could protect kidney tissue against

Cd-induced damage, as LDH activity and protein content in urine were significantly reduced. CPZ could not promote Cd excretion from urine, however, it can not be excluded that Cd excretes from other ways such as feces. Chronic administration of CPZ can decrease PKC activity in the membrane fraction of cortex, hippocampus, and striatum. In this study, it was also found that PKC activity in the cytoplasm of renal cortex significantly decreased in CPZ-pretreatment group. CPZ-pretreatment suppressed the elevated activities of PKC, Na^+-K^+-ATPase and Ca^{2+}-ATPase in renal cortex. The experimental data also showed that Ver restrained Cd^{2+} absorption. Ver was as effective as CPZ in suppressing the nephrotoxicity of Cd, which reduced LDH activity and protein level in urine and the activities of PKC, Na^+-K^+-ATPase and Ca^{2+}-ATPase in renal cortex. The results indicated that the CPZ and Ver partly protected against toxic effects induced by Cd.

In conclusion, the present results show that Cd could activate the activities of PKC, Na^+-K^+-ATPase and Ca^{2+}-ATPase. Moreover, CPZ- and Ver-pretreatment could reduce nephrotoxicity of Cd. Its possible mechanism is that CPZ and Ver can block Ca^{2+} influx and activation of CaM so as to reduce renal damage. However, the exact mechanism of nephrotoxicity of Cd need still further studies.

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氯丙嗪和维拉帕米对大鼠镉肾毒性的影响

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摘要：目的　研究氯丙嗪(CPZ)和维拉帕米(Ver)对由镉引起的大鼠肾毒性是否有预防作用。**方法**　32只大鼠随机分成4组，分别为对照组、单纯染镉组、CPZ和Ver预处理组。单纯染镉组大鼠 sc 7 μmol·kg⁻¹氯化镉；CPZ和Ver预处理组分别 ip CPZ 5 mg·kg⁻¹和Ver 4 mg·kg⁻¹，1 h后 sc 7 μmol·kg⁻¹氯化镉；对照组在相应时间内给予生理盐水，注射容量均为2 mL·kg⁻¹。最后一次注射24 h后，收集24 h尿样，测定尿乳酸脱氢酶(LDH)活性、尿蛋白、尿镉、肾镉和肾皮质中的 Na^+-K^+-ATP酶，Ca^{2+}-ATP酶和蛋白激酶C(PKC)的活性。**结果**　单纯染镉组与对照组比较，尿镉和肾镉含量明显升高。CPZ和Ver预处理组尿镉明显低于单纯染镉组，但肾镉无明显变化。与对照组比较，单纯染镉组尿LDH活性、尿蛋白和肾皮质中的 Na^+-K^+-ATP酶，Ca^{2+}-ATP酶和PKC活性明显升高。CPZ和Ver预处理组大鼠尿LDH活性、尿蛋白和肾皮质中的 Na^+-K^+-ATP酶，Ca^{2+}-ATP酶和PKC活性明显低于单纯染镉组。**结论**　镉能激活 Na^+-K^+-ATP酶，Ca^{2+}-ATP酶和PKC的活性，而且，CPZ和Ver均可不同程度地减轻肾毒性。

关键词： 镉；氯丙嗪；维拉帕米；肾毒性

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幼年家兔口服苯妥英锌7个月的小脑生长发育

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苯妥英钠(phenytoin sodium，PS)虽是治疗除失神发作外各型癫痫病的首选药物之一，但长期服用后可引起小脑萎缩。苯妥英锌(phenytoin zinc，PZ)的抗癫痫作用及急性和亚急性毒性与PS相近，但大鼠长期口服后对脑重量的影响正好与PS相反，提示PZ长期服用后有可能减轻小脑萎缩。本研究选用1月龄家兔35只，随机分为对照组，PZ或PS 10和30 mg·kg⁻¹·d⁻¹组。给药前进行体重、血常规检查。动物单笼饲养，连续 *po* 7个月。服药前、服药4个月和服药结束时进行CT扫描，以兔外耳门为扫描基线，扫描架向头侧倾斜7～10°，连续扫描11层，扫描时间、厚度和间距分别为1 s，5 mm和5 mm。以颈突为平面，测量整个小脑的最大横径；以外听道前缘为平面，测量小脑中央蚓部最大横径，同时在下位扫描层面观察四脑室、中央管的形态变化。服药结束后，分离全脑并称重，用70%乙醇脱水保存在4℃备用。1个月后测定大脑和小脑的长度、宽度和高度，以及蚓部的长度和宽度，称重并计算脑的脏器重指数。结果表明，PS 30 mg·kg⁻¹组服药4个月后，小脑最大横径(15.8±2.1)mm比对照组(18.3±2.2)mm缩短14.0%($P<0.05$)，蚓部最大横径(11.8±1.6)mm比对照组(14.2±1.9)mm缩短16.4%($P<0.05$)；服药7个月后，小脑最大横径(13.2±2.5)mm比对照组(19.5±2.2)mm缩短32.2%($P<0.01$)，蚓部最大横径(9.8±1.4)mm比对照组(14.2±1.8)mm缩短30.8%($P<0.01$)。PZ 30 mg·kg⁻¹组服药4个月后小脑和蚓部最大横径与对照组比较无显著性差异；服药7个月小脑最大横径(16.4±1.7)mm比对照组(19.5±2.2)mm缩短16.1%($P<0.05$)，蚓部最大横径(11.4±1.8)mm比对照组(14.2±1.8)mm缩短19.7%($P<0.05$)。PZ和PS对大脑径线，四脑室和中央管的形态学未见明显改变。PS 30 mg·kg⁻¹组小脑长度(14.6±0.4)mm和宽度(18.1±0.7)mm均比对照组(15.3±0.4)和(19.5±0.7)mm显著缩小($P<0.01$)，蚓部长度(11.1±0.6)mm和宽度(5.7±0.6)mm也比对照组(12.2±0.5)和(6.4±0.4)mm明显缩小($P<0.05$)。PZ 30 mg·kg⁻¹组除小脑宽度(18.6±0.8)mm比对照组(19.5±0.7)mm明显缩小外($P<0.05$)，其他各测量值与对照组比较差异无显著性。PS 30 mg·kg⁻¹组全脑湿重比对照组减轻10.0%($P<0.05$)，全脑脱水后重量比对照组减轻15.4%($P<0.05$)，小脑脱水后重量比对照组减轻18.4%($P<0.05$)；PZ 30 mg·kg⁻¹组全脑湿重、全脑脱水重及小脑脱水重无显著性差异。结果表明，幼年家兔口服PZ 30 mg·kg⁻¹ 4个月或PZ 10 mg·kg⁻¹ 7个月不会引起小脑明显萎缩。

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