

Evaluation of the effect of glutathione on cisplatin antitumor activity and kidney injury at different administration times

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Abstract. Cisplatin (cis-diamminedichloroplatinum, CDDP) is one of the most potent anticancer drugs. However, the therapeutic value of CDDP is greatly compromised by its dose-limiting nephrotoxicity. This study was performed to investigate whether reduced glutathione (GSH) was able to reduce the kidney injury induced by CDDP and whether it affected the anticancer activity of CDDP *in vivo* and *in vitro*. In *in vivo* experiments, mice were divided into five groups: control, CDDP only and three GSH treatment groups. Blood samples were collected 72 h after CDDP administration to determine the levels of blood urea nitrogen (BUN) and plasma creatinine (Cr). In addition, we examined antioxidative parameters, malondialdehyde (MDA) levels and histopathological changes in the kidney. In order to investigate whether GSH affected the anticancer activity of CDDP, we performed a sulforhodamine B (SRB) assay to determine the anti-proliferative effect in three tumor cell lines of treatment with CDDP alone or combined with GSH and examined the cell morphology. The results revealed that GSH decreased the BUN and Cr levels in plasma, ameliorated the pathological changes induced by CDDP and enhanced the endogenous antioxidant capacities in all three GSH groups. Furthermore, GSH significantly inhibited the growth of the three tumor cell lines when combined with CDDP and did not affect the inhibitory effect of CDDP on the carcinoma cell proliferation. In addition, we found no differences among the three GSH groups. These findings suggest that GSH is able to attenuate the nephrotoxicity induced by CDDP, not only when administered prior to CDDP, but also when administered at the same time as or subsequent to CDDP administration, without affecting the

anticancer activity of CDDP. Thus, the administration of GSH is a promising approach for attenuating the nephrotoxicity caused by CDDP.

Introduction

Cisplatin (cis-diamminedichloroplatinum, CDDP) is a chemotherapeutic drug used to treat various types of cancer, including sarcomas, certain carcinomas, such as small cell lung and ovarian cancers, lymphomas, and germ cell tumors (1). CDDP reacts *in vivo*, binding to and causing crosslinking of DNA, which ultimately triggers apoptosis/programmed cell death (2). Despite its desirable anticancer therapeutic value, CDDP-based chemotherapy has a number of dose-limiting side-effects, the most severe being nephrotoxicity (3,4). The glomerular filtration rate may decrease by 30% after only two doses, often making the continuation of the treatment implausible (5). The CDDP-induced alterations in kidney function are characterized by signs of injury, including changes in glutathione (GSH) status and lipid peroxidation (LPO) levels (6). Therefore, the investigation of reagents that can reduce the nephrotoxicity of CDDP without affecting its anticancer effectiveness is critical for the successful administration of the drug and the survival rates of cancer patients.

GSH has been shown to be an effective protector against CDDP-induced nephrotoxicity in various rodent models. Zunino *et al* (7) found that GSH had a protective effect against CDDP-induced renal toxicity only when administered prior to CDDP and the mechanisms of the protective effect were not clear. The present study expands previous observations by investigating the protective effect of GSH against CDDP-induced renal toxicity when administered at various times and exploring its mechanisms of action. Additionally, whether GSH affected the antitumor effect of CDDP in the three tumor cell lines was also investigated. Oxidative stress has been reported in CDDP-induced nephrotoxicity. Kidneys contain antioxidant enzymes, including superoxide dismutase (SOD), lipid peroxides and GSH, which protect the kidney from free radicals and superoxides. Subsequent to CDDP administration, GSH levels as well as the activities of SOD and GSH-peroxidase (GSH-Px) are decreased (8). GSH is a tripeptide compound containing a sulfhydryl (thiol) group. As with

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all thiol group-containing molecules, GSH is an antioxidant, preventing the damage to important cellular components caused by reactive oxygen species, including free radicals and peroxides (9). GSH has no known toxicity. Clinically, it is used as a supplementary antineoplastic drug, but its half-life when administered intravenously (*i.v.*) is only 15 min. Thus, to avoid GSH decreasing the effect of anticancer drugs, it is most commonly administered prior to CDDP and is administered continuously for 15-20 min. To the best of our knowledge, the best method of administering GSH, the existence of any differences between the effects of the different GSH administration methods and whether GSH is able to protect against the kidney injury induced by CDDP, have not been reported. The aim of this study was to investigate whether reduced GSH is capable of reducing the kidney injury induced by CDDP and whether it affects the anticancer activity of CDDP *in vivo* and *in vitro*.

Materials and methods

Chemicals and instruments. CDDP was obtained from Qilu Pharmaceutical Co., Ltd. (Shandong, China). GSH was supplied by Shandong Luye Pharmaceutical Co., Ltd. (Yantai, China). RPMI-1640 cell culture medium was purchased from Gibco-BRL (Grand Island, NY, USA). Sulforhodamine B (SRB) was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Blood urea nitrogen (BUN), creatinine (Cr), SOD, GSH-Px and malondialdehyde (MDA) test kits were purchased from the Jiancheng Bioengineering Institute (Nanjing, China). Any other chemicals and reagents used in this study were of analytical grade. GSH was diluted with normal saline. The TDL-40B refrigerated centrifuge was obtained from the Shanghai Anting Scientific Instrument Factory (Shanghai, China). The Synergy HT Microplate Reader was from BioTek Instruments, Inc. (Winooski, VT, USA). The FJ-200S high-speed tissue homogenate machine was from the Shanghai Specimen and Model Factory (Shanghai, China).

Animals and drug treatment. Adult female Swiss mice weighing 22-28 g were obtained from the Experimental Animal Center of Shandong Province (Certificate no. 20041225). All experimental procedures carried out in this study were performed in accordance with the guidelines for the care and use of laboratory animals of Yantai University and were approved by the Ethics Committee. The mice were kept with free access to food and water on a 12-h light/dark cycle. The animals were housed in plastic cages and randomly assigned to 5 groups as follows: control, CDDP-intoxicated (20 mg/kg *i.v.*), GSH-treated 1 (pretreated with GSH at doses of 1,200 mg/kg *i.v.* for three consecutive days), GSH-treated 2 (treated with GSH at doses of 1,200 mg/kg *i.v.* 30 min subsequent to CDDP intoxication) and GSH-treated 3 (treated with GSH at doses of 1,200 mg/kg *i.v.* for three consecutive days subsequent to CDDP intoxication). Each group contained 10 animals and the animals in the control group were injected with normal saline in equivalent volumes. Seventy-two hours after a single injection of CDDP, the acute kidney injury model was established, the mice were sacrificed and blood samples were collected from the orbital venous plexus for determination of

plasma BUN and Cr levels. Kidneys were removed for the determination of SOD and GSH-Px activities, MDA levels and for histological examination.

Histopathology. Paraformaldehyde (4%)-fixed and paraffin-embedded kidney samples were cut into 4- μ m sections, deparaffinized in xylene and rehydrated through a series of decreasing concentrations of ethanol. Sections were stained with hematoxylin and eosin. Pathological observation of the tissue was performed by light microscopy.

Biochemical analysis. On the day of the sacrifice, blood was collected immediately and blood samples were drawn into heparinized tubes for biochemical analysis. The samples were centrifuged immediately (3500 x g for 10 min at 4°C), and the plasma was stored at -80°C.

The kidney was homogenized in ice-cold normal saline (1/9, w/v) at a speed of 5000 rpm (5x15 sec, in the ice water bath). The suspension was centrifuged at a speed of 2000 rpm for 10 min at 4°C and the supernatant was stored at -80°C.

The BUN and Cr levels of the plasma and the SOD and GSH-Px activities and MDA levels in the kidney homogenate supernatants were determined according to the instructions of the respective test kits. Antioxidant enzyme activities and MDA levels were expressed as U/mg protein and were determined by the Coomassie Blue method.

Cell lines. The lung (A549) and gastric (SGC-7901) carcinoma cell lines, as well as the breast cancer cell line (MCF-7) were obtained from the School of Pharmacy, Yantai University. The tumor cells were routinely maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. The cells were cultured at 37°C, under an atmosphere containing 5% CO₂ at 95% relative humidity.

Cytotoxicity assays. Cytotoxicity was evaluated by an SRB assay using 96-well plates (10). In the SRB assay, the cell suspension (100 μ l) was plated at a density of 5x10⁴ cells/ml and incubated for 24 h. The medium was removed and replaced with drugs. The plates were then allowed to incubate for up to 48 h and the cells were fixed with 50% (w/v) trichloroacetic acid (TCA) for 1 h at 4°C. After washing with distilled H₂O to remove TCA, the cell layer was stained with 0.4% (w/v) SRB in 1% acetic acid for 30 min. After extensive washing with 1% (v/v) acetic acid, the protein-bound dye was dissolved in 10 mM Tris base solution and the optical density was determined at 515 nm. Duplicate plates containing the single agent, the combination and the control groups were subjected to the SRB assay. The inhibition rate (%) was calculated as $(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100\%$, where A_{control} is the control absorbance and A_{sample} is the test sample absorbance. The groups and administration quantities used to treat the three tumor cell lines are shown in Table I.

Cell morphology. The A549, SGC-7901 and MCF-7 cells were exposed to fresh medium, CDDP, GSH and CDDP combined with GSH for 24 h as described in the cytotoxicity assays. The cell morphology changes of each group were observed using an inverted microscope.

Table I. Groups and administration in cytotoxicity assays.

Group	Fresh medium (μl)	GSH 60 $\mu\text{g/ml}$ (μl)	GSH 120 $\mu\text{g/ml}$ (μl)	CDDP 6 $\mu\text{g/ml}$ (μl)
Control	200	-	-	-
GSH (30 $\mu\text{g/ml}$)	100	100	-	-
GSH (60 $\mu\text{g/ml}$)	100	-	100	-
CDDP (3 $\mu\text{g/ml}$)	100	-	-	100
GSH + CDDP (30 + 3 $\mu\text{g/ml}$)	-	100	-	100
GSH + CDDP (60 + 3 $\mu\text{g/ml}$)	-	-	100	100

GSH, glutathione; CDDP, cisplatin.

Acridine orange/ethidium bromide (AO/EB) fluorescence staining. Cellular morphological changes were investigated by AO/EB staining using fluorescence microscopy. The A549 cells were exposed to fresh medium, CDDP, GSH and CDDP combined with GSH for 24 h. Cells from the various groups were collected and prepared into single-cell suspensions and the cell concentration was adjusted to $1 \times 10^7/\text{ml}$ with PBS buffer. Adhesive and suspended cells were collected, pooled, pelleted and resuspended in 200 μl medium. An 8- μl mixture of fluorescent dyes containing 100 $\mu\text{g/ml}$ AO and 100 $\mu\text{g/ml}$ EB (Sigma) was then added to the cells and mixed gently. A drop of the mixture was placed on a microscope slide and covered with a coverslip. The cells were visualized under a fluorescent microscope (x100) (Nikon, Tokyo, Japan), using a blue filter.

Statistical analysis. One-way ANOVA was used to analyze the significant differences among the various groups. Comparisons between the two groups were determined by the Student's unpaired t-test, using SPSS 11.5 statistical software. $P < 0.05$ was considered to indicate a statistically significant result. The renal cortical slices for each experiment were from at least three mice. Data in this study were expressed as the mean \pm SD.

Results

Effect of GSH on the histopathologic changes in the kidney induced by CDDP. In the control group, the renal cortical sections were normal (Fig. 1a). CDDP treatment resulted in necrosis of the proximal tubules at various foci throughout the cortex, karyomegaly, hyaline casts in tubular lumen, desquamation and parenchymal degeneration of the tubular epithelium cells (Fig. 1b). Treatment with GSH (1,200 mg/kg) at various times significantly improved the CDDP-induced renal tubular necrosis and degenerative changes (Fig. 1c-e).

Effect of GSH on the plasma BUN and Cr levels in mice treated with CDDP. The levels of BUN and Cr were measured 72 h after the administration of CDDP. As shown in Fig. 2A and B, CDDP administration induced severe renal dysfunction, which was clearly attenuated by GSH and no

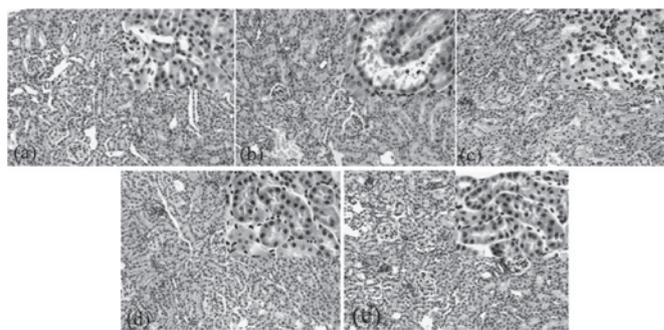


Figure 1. Effect of glutathione (GSH) on histopathologic changes in kidney induced by cisplatin (CDDP). (a) Control, (b) CDDP, (c) GSH1, (d) GSH2 and (e) GSH3 groups.

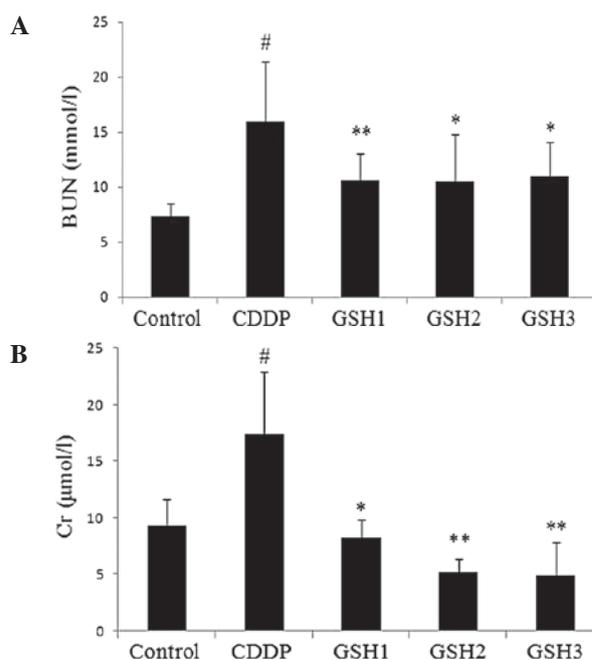


Figure 2. Effect of glutathione (GSH) on plasma (A) blood urea nitrogen (BUN) and (B) creatinine (Cr) contents in CDDP-intoxicated mice (n=8). # $P < 0.01$ compared with the control group; ** $p < 0.01$, * $p < 0.05$, compared with the cisplatin (CDDP)-treated group.

differences were observed among the three GSH groups. The BUN and Cr levels in the control mice prior to the injection of CDDP were 7.34 mmol/l and 9.40 $\mu\text{mol/l}$, respectively. The injection of CDDP (20 mg/kg) produced an elevation of the BUN and Cr levels ($p < 0.01$). The GSH-treated groups had markedly decreased BUN and Cr levels 72 h after CDDP administration ($p < 0.01$, $p < 0.05$).

Effect of GSH on the SOD and GSH-Px activities and MDA levels in the kidney subsequent to CDDP administration. The activity of SOD in the kidney was significantly decreased 72 h after CDDP administration ($p < 0.01$). The GSH treatment (1,200 mg/kg) increased the SOD activity 72 h later ($p < 0.01$, $p < 0.05$) (Fig. 3A). The activity of GSH-Px in the kidney was also decreased 72 h after CDDP administration ($p < 0.01$). In addition, the GSH treatment (1,200 mg/kg) increased GSH-Px activity significantly 72 h later ($p < 0.01$, $p < 0.05$) (Fig. 3B). The MDA levels in the kidney were significantly increased 72 h

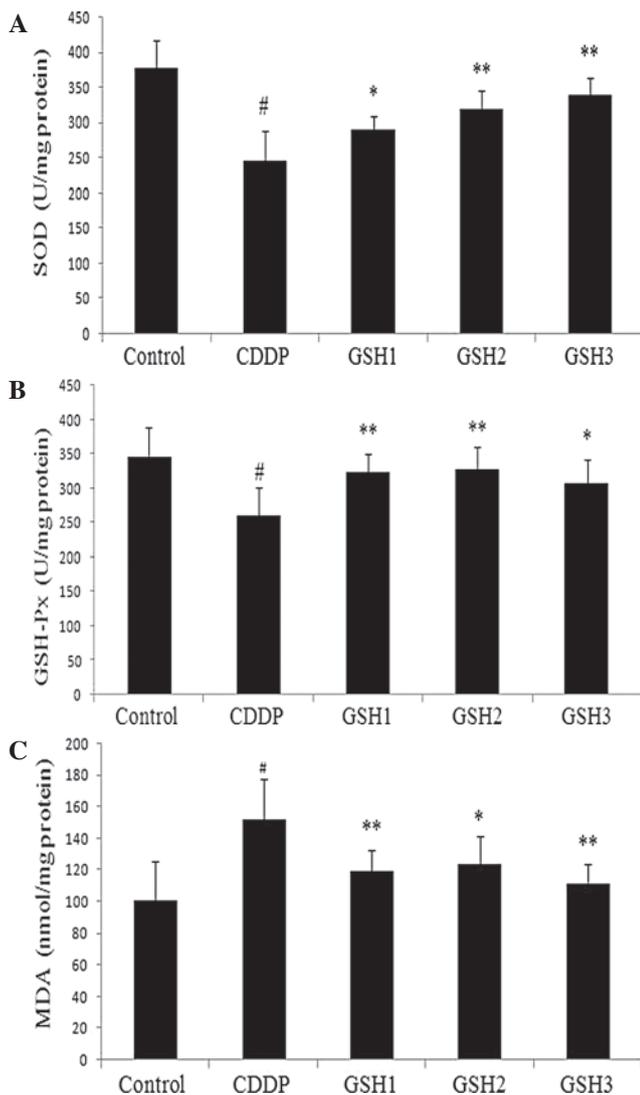


Figure 3. Effect of glutathione (GSH) on (A) superoxide dismutase (SOD) and (B) GSH-peroxidase (GSH-Px) activities and (C) malondialdehyde (MDA) levels in kidney following cisplatin (CDDP)-intoxication (n=8). [#]P<0.01 compared with the control group; ^{**}p<0.01, ^{*}p<0.05 compared with the CDDP-treated group.

after CDDP administration (p<0.01). The GSH treatment (1,200 mg/kg) decreased the MDA levels 72 h later (p<0.01, p<0.05) (Fig. 3C).

The antiproliferative effects of CDDP alone or combined with GSH on cancer cells. The SRB assays demonstrated that CDDP combined with GSH significantly inhibited the proliferation of A549, SGC-7901 and MCF-7 cells. No significant differences were observed between the antiproliferative rates in the cancer cells of the GSH groups and the respective controls (p>0.05) or between the low and high GSH concentration groups (p>0.05). The CDDP group was not observed to have any significant differences from the CDDP and GSH combination groups (p>0.05) (Table II).

Morphological observation of tumor cells. Morphological observation was carried out using an inverted microscope. The results revealed the profiles of three tumor cell lines in the GSH and control groups to be clear with the cells growing

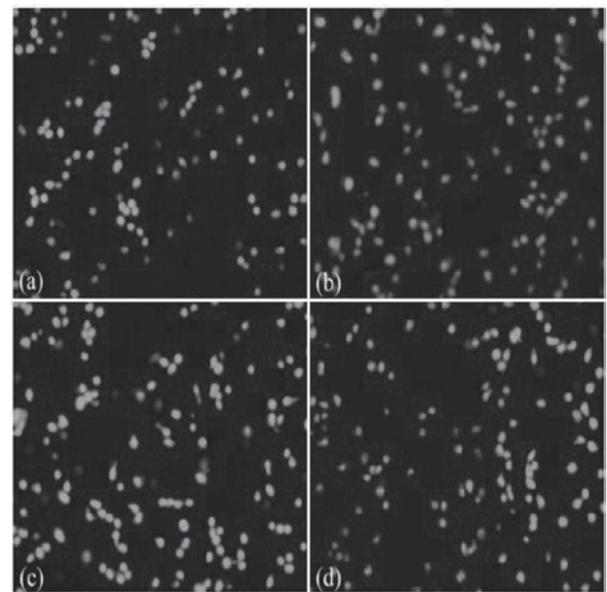


Figure 4. Observation using orange/ethidium bromide (AO/EB) fluorescence staining. (a) Control. (b) glutathione (GSH), (c) cisplatin (CDDP) and (d) CDDP + GSH groups. Cells in the control group received fresh medium (200 μ l). The GSH group received 100 μ l fresh medium and 100 μ l fresh medium containing GSH (60 μ g/ml). The CDDP group received 100 μ l fresh medium and 100 μ l fresh medium containing CDDP (6 μ g/ml). The CDDP + GSH group received fresh medium containing 100 μ l GSH (60 μ g/ml) and 100 μ l CDDP (6 μ g/ml).

adherently in a polygonal shape. Subsequent to 24-h single- and combined-drug treatments, the cell density in the CDDP treatment and combination treatment groups decreased significantly and the volume of the cytoplasmic vesicles appeared larger than in the control and GSH groups. Apoptotic bodies and cell morphology changes were evident. Cells decreased in size and became rounded and smaller. The adherence capacity declined, while most cells dropped off and were floating. No significant differences were observed among the CDDP and the combination groups.

Detection of apoptosis using AO/EB fluorescence staining. AO is a cell-permeable dye that intercalates into DNA, resulting in a color change. EB enters cells with disrupted membranes and intercalates into RNA and double-stranded DNA. Thus, the differential uptake and binding of these dyes allowed us to identify cells in the early and late stages of apoptosis and necrosis.

In the CDDP and combination groups, when viewed under a fluorescent microscope, early apoptotic cells showed the presence of patches of fragmented and condensed chromatin and late apoptotic cells showed the presence of patches of fragmented and condensed chromatin (Fig. 4). No significant differences were observed among the CDDP and combination groups.

Discussion

CDDP is one of the most potent antitumor agents against a diverse spectrum of malignancies. However, the use of this agent in combating cancer is limited by its nephrotoxicity since it preferentially accumulates in human kidney cells (11). The

Table II. Antiproliferative effects of CDDP alone or with GSH on cancer cells.

Group	A549			SGC-7901			MCF-7		
	$\bar{x} \pm SD$	F	p-value	$\bar{x} \pm SD$	F	p-value	$\bar{x} \pm SD$	F	p-value
GSH (30 $\mu\text{g/ml}$)	2.363 \pm 0.084			0.796 \pm 0.095			1.823 \pm 0.068		
GSH (60 $\mu\text{g/ml}$)	2.395 \pm 0.134	1.109	>0.05	0.722 \pm 0.134	0.490	>0.05	1.798 \pm 0.106	1.374	>0.05
Control group	2.312 \pm 0.054			0.751 \pm 0.082			1.885 \pm 0.041		
GSH (30 $\mu\text{g/ml}$) + CDDP	0.377 \pm 0.032			0.148 \pm 0.013			0.692 \pm 0.020		
GSH (60 $\mu\text{g/ml}$) + CDDP	0.398 \pm 0.043	2.003	>0.05	0.133 \pm 0.009	1.036	>0.05	0.699 \pm 0.017	0.947	>0.05
CDDP	0.333 \pm 0.084			0.152 \pm 0.031			0.710 \pm 0.021		

Values are given as the mean \pm SD. Cells in the control group received 200 μl fresh medium. GSH 30 and 60 $\mu\text{g/ml}$ groups received 100 μl fresh medium and 100 μl fresh medium containing GSH 60 and 120 $\mu\text{g/ml}$, respectively. CDDP group received 100 μl fresh medium and 100 μl fresh medium containing CDDP (6 $\mu\text{g/ml}$). GSH (30 $\mu\text{g/ml}$) + CDDP and GSH (60 $\mu\text{g/ml}$) + CDDP groups received 100 μl fresh medium containing GSH (60 $\mu\text{g/ml}$) and 100 μl CDDP (6 $\mu\text{g/ml}$) or 100 μl GSH (120 $\mu\text{g/ml}$) and 100 μl CDDP (6 $\mu\text{g/ml}$), respectively. $P > 0.05$ indicates no significant differences among the groups. GSH, glutathione; CDDP, cisplatin.

primary target of CDDP in the kidney is the S3 subsegments of the proximal tubular epithelial cells (12), where CDDP accumulates and causes cellular damage (13). CDDP-induced nephrotoxicity is associated with increased renal vascular resistance and morphological damage to the intracellular organelles, including cellular necrosis, loss of microvilli, changes in the number and size of lysosomes and mitochondrial vacuolization (14). There is a continuous search for agents that provide nephroprotection against CDDP and other platinum drugs, including antioxidants, modulators of nitric oxide, diuretics and cytoprotective and apoptotic agents (15). However, none of these agents have been found to be suitable for clinical use in the protection against CDDP-induced nephrotoxicity.

GSH is a vital nutrient for normal human cells. Currently, GSH is administered as a supplementary antineoplastic drug to mitigate the damage caused by chemotherapy. However, whether GSH acts as a nutrient to cancer cells or affects the anticancer activity of CDDP is not clear. Furthermore, in clinical use, GSH is usually administered prior to CDDP in order to avoid a nutritional effect on the tumor cells. Whether GSH may protect the kidney from injury, when administered following or at the time of CDDP administration, and its mechanism, are not clear. In this study, we demonstrated that the antioxidant GSH was able to attenuate the nephrotoxicity induced by CDDP, not only when administered prior to CDDP, but also when administered following or at the time of CDDP administration. In addition, GSH did not affect the anticancer activity of CDDP.

In vivo, we found that exogenously administered GSH effectively reduced nephrotoxicity in the mice treated with CDDP, as supported by the following evidence. Firstly, GSH improved the kidney's function in the clearance of BUN and Cr in the CDDP-treated mice. Secondly, GSH decreased the renal production of MDA in the CDDP-treated mice. Thirdly, GSH restored SOD and GSH-Px activities in the CDDP-treated mice. Therefore, the data suggest that GSH is effective in counteracting the nephrotoxic side effects of CDDP, while

the three administration methods were observed to have no significant differences from each other.

However, a major concern remains with regard to whether GSH is likely to be a nutrient to tumor cells and affect the efficacy of CDDP in the treatment of cancer. Using the SRB assay, we showed that CDDP, whether administered alone, or combined with GSH of different concentrations, inhibited A549, SGC-7901 and MCF-7 tumor cell line proliferation to a similar extent, indicating that, at the concentrations tested, GSH has no significant effect on the cytotoxicity of CDDP towards tumor cells. GSH combined with CDDP may significantly arrest A549 cells in the G0/G1 phase and induce apoptosis to a certain extent (16). Under a fluorescent microscope, in the CDDP and the combination groups, early apoptotic cells showed the presence of green patches of fragmented and condensed chromatin and late apoptotic cells showed the presence of orange patches of fragmented and condensed chromatin. In comparison, viable cells were uniformly green and necrotic cells were uniformly orange (Fig. 4). The CDDP and combination groups were not observed to have any significant differences. This result is rather noteworthy, revealing that GSH differentially protects the normal cells from cytotoxicity but not the tumors.

CDDP therapy induces oxidative stress, largely involving ROS, including superoxide anions, hydrogen peroxide and hydroxyl radicals (17) in renal tubular cells (18). The decrease in antioxidant enzymes, including GSH-Px and SOD, further deteriorates the situation by allowing more ROS to accumulate. The interaction of ROS with various cellular components may cause damage to DNA, proteins and lipids. The excessive ROS accumulation caused by CDDP may overwhelm the natural antioxidant defenses of the kidney cells and lead to LPO and delayed-onset kidney injury (19).

GSH reduces H_2O_2 and LPO, and these reductions are catalyzed by GSH-Px (11). The early depletion of GSH following CDDP treatment appears to be necessary for the CDDP-induced LPO and subsequent toxicity. The decreased

SOD activity may also cause the initiation and propagation of LPO in the CDDP-treated mice. The MDA concentration increases as a result of LPO. In our study, the GSH supplementary treatment consistently reversed the decrease in SOD and GSH-Px activities and the increase in MDA levels in the CDDP-treated mice, suggesting that the nephroprotective effect of GSH is partially mediated by the prevention of the CDDP-induced decline of renal antioxidant status.

In conclusion, our study has demonstrated that GSH has no effect on the cytotoxicity of CDDP to tumor cells and is able to attenuate the CDDP-induced nephrotoxicity in mice. We also confirmed that when administered at various times, GSH does not reduce the antitumor effects of CDDP and so should be beneficial to patients undergoing CDDP chemotherapy. These observations have clear clinical implications in future studies of GSH as a protector against CDDP toxicity.

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