


# Protective Effect of D-Methionine on Body Weight Loss, Anorexia, and Nephrotoxicity in Cisplatin-Induced Chronic Toxicity in Rats

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## Abstract

D-methionine is a sulfur-containing amino acid that can act as a potent antioxidant. Anorexia and nephrotoxicity are side effects of cisplatin. The protective effects of D-methionine on cisplatin-induced anorexia and renal injury were investigated. The model of chronic cisplatin administration (5 mg/kg body weight) involved intraperitoneal injection on days 1, 8, and 15 and oral D-methionine (300 mg/kg body weight) coadministration daily for 20 days. On the 21st day of treatment, food intake and body weight in the cisplatin-treated group significantly decreased by 52% and 31%, respectively, when compared with a control group. D-methionine coadministration with cisplatin decreased food intake and body weight by 29% and 8%, respectively. In cisplatin-treated rats, white blood cell, mean corpuscular volume, and platelet values significantly decreased, while mean corpuscular hemoglobin concentration significantly increased by 8.6% when compared with control rats. Cisplatin administration resulted in significantly decreased feeding efficiency, elevated renal oxidative stress, and reduced antioxidative activity. Leukocyte infiltration, tubule vacuolization, tubular expansion, and swelling were observed in the kidneys of cisplatin-treated rats. Oral D-methionine exhibited an antianorexic effect, with improvement in food intake, feeding efficiency, and hematological toxicities, as well as a protective effect against nephrotoxicity by elevated antioxidative activity. D-methionine may serve as a chemoprotectant in patients receiving cisplatin as part of a chemotherapy regimen.

## Keywords

cisplatin, D-methionine, anorexia, nephrotoxicity, chemotherapy

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## Introduction

One of the major treatments for malignant solid tumors is chemotherapy. However, antineoplastic agent efficacy is usually limited due to toxic side effects. Cisplatin, cisplatinum, or cis-diamminedichloroplatinum (II) is a common clinical anticancer drug. Unfortunately, peripheral neuropathies, myelosuppression, gastrointestinal toxicity, nephrotoxicity, hepatotoxicity, and ototoxicity induced by cisplatin have been widely reported.<sup>1,2</sup> Cisplatin causes extremely undesirable side effects, which increase morbidity and reduce quality of life, including dyspepsia, also known as indigestion or upset stomach, a symptom that includes bloating, nausea, and burping. Cisplatin-induced gastrointestinal tract disorders can include acute and delayed nausea and vomiting, gastric stasis, reduced food intake, and subsequent weight loss.<sup>3–8</sup> The most common clinical sign in patients suffering from cancer is weight loss, especially after cisplatin treatment,<sup>9</sup> which can contribute to decreased

clinical recommended dose and even compel patients to give up treatment. The dosage of antineoplastic agent treatment and the tumor itself can be risk factors for weight loss.<sup>10</sup> The incidences of weight loss and malnutrition are 31% to 87%, respectively, in patients with cancer.<sup>11</sup> Weight loss induced by cisplatin is frequently found in either clinical trials or acute/chronic animal models.<sup>5–7,12,13</sup> Despite antineoplastic agent effectiveness, results of clinical trials have demonstrated that approximately 58% of cancer patients experience some degree of weight loss.<sup>14</sup> The incidence of anorexia, defined as the loss of the desire to eat, is

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approximately 15% among patients with cancer receiving high-dose cisplatin treatment.<sup>15</sup> A novel and feasible treatment approach is essential to improving the outcome of anorexia and the negative effect of anorexia on quality of life, as well as the ability to cope with the cancer.

To maintain or even enhance clinical effectiveness, various compounds have been used to ameliorate weight loss caused by chemotherapy.<sup>7,10,16-18</sup> We further calculated the molar ratios from these cited references and found that the molar ratios of D-methionine and Ghrelin, respectively, relative to cisplatin are 38:1,<sup>12</sup> 121:1,<sup>19</sup> and 0.63:1,<sup>7</sup> which could be effective in protecting animals from the toxicity of cisplatin-induced weight loss. Oxidative stress is involved in cisplatin-induced adverse effects and antioxidants including curcumin, selenium, vitamin E, and polydatin (a natural precursor of resveratrol) are frequently applied by oral administration as protective agents against cisplatin-induced toxicity.<sup>20-23</sup> The molar ratios of these compounds relative to cisplatin were as follows: curcumin–cisplatin (8.2:1), selenium–cisplatin (0.95:1), vitamin E–cisplatin (116:1), and polydatin–cisplatin (2.5-10:1).

D-methionine is a sulfur-containing nucleophile that may act as both a direct and indirect antioxidant. Campbell et al first demonstrated that D-methionine provides protection against cisplatin-induced ototoxicity in rats.<sup>12</sup> D-methionine's otoprotective mechanisms have been deeply and widely investigated with focus on specific antioxidant enzyme activities and oxidative status.<sup>22,24,25</sup> A previous *in vitro* and *in vivo* study has suggested that D-methionine is a potential protector against radiation and cisplatin combined-induced oral mucositis, but does not alter response to therapy.<sup>26</sup> However, the number of studies performed on the protective effect of D-methionine on cisplatin-induced weight loss and anorexia is limited.<sup>12</sup> In a short-term animal study, 300 mg/kg D-methionine administered 30 minutes before 16 mg/kg cisplatin infusion (molar ratio 38:1, D-methionine–cisplatin) reduced weight loss and improved survival.<sup>12</sup> To date, the effects of D-methionine on anorexia caused by cisplatin are not completely understood. Recent studies have suggested that administration of low-dose cisplatin once a week for several weeks is more clinically relevant and allows for observation of long-term toxicity.<sup>27</sup> The purpose of this study is to determine if D-methionine, a sulfur-containing antioxidant, protects against cisplatin-induced anorexia, weight loss, and nephrotoxicity in a chronic administration model and whether the beneficial effect of D-methionine is associated with anti-inflammatory/antioxidant activities.

## Materials and Methods

### Drugs and Chemicals

Cisplatin and D-methionine were purchased from Sigma-Aldrich (St Louis, MO). All other chemicals and reagents used in this study were of analytical grade.

### Animals

Six-week-old male Wistar rats were purchased from BioLASCO Taiwan Co, Ltd. Rats were housed in cages with a maximum of 4 rats per cage with a 12-hour light/12-hour dark cycle and fed an autoclaved diet with *ad libitum* access to standard rodent chow (LabDiet, 5001) during the study period. After 2 weeks of acclimatization, animals were randomly divided into 3 equal groups consisting of 7 animals each: (1) Control group: distilled water was administered by gavage daily from day 1 and 0.9% saline was administered by intraperitoneal (ip) injection on days 1, 8, and 15; (2) Cisplatin group: cisplatin (5 mg/kg of body weight) was administered by a single ip injection to induce cisplatin toxicity on days 1, 8, and 15 with distilled water administered by gavage in the same volumes; And (3) Cisplatin + D-methionine group: D-methionine was diluted in phosphate-buffered saline (PBS) and administered by gavage at 300 mg/kg weight/day per rat. The dose of either cisplatin or D-methionine was used and based on the published literature.<sup>12,24</sup> In our study, the molar ratio of D-methionine to cisplatin is 121:1. Vuyyuri et al had demonstrated that D-methionine protected normal cells from radiation and cisplatin combined-induced mucosal injury in a murine model. And the molar ratio of D-methionine to cisplatin (251:1) did not significantly interfere with the antitumor efficacy of cisplatin.<sup>26</sup> Previous studies have shown that the dose of cisplatin (5 mg/kg) was sufficient to induce nephrotoxicity in rats.<sup>28</sup> In our study, the dose of cisplatin (5 mg/kg) compared to the human clinical dose in mg/m<sup>2</sup> was calculated using a K<sub>m</sub> factor, which is the ratio of weight to surface area for an adult rat.<sup>29</sup> The corresponding dose in human being (60 kg) is 35 mg/m<sup>2</sup>, which is in the therapeutic range for cisplatin use in clinical practice.

The rationale for choosing a 20-day experimental period was to evaluate the impact of repeated treatment with cisplatin, which resembles the human clinical regimen, on gastrointestinal dysfunction and nephrotoxicity in a rat model. Each animal's body weight, food intake, and water consumption were recorded daily during the experiments. All animals were sacrificed on day 21. Blood samples were obtained with some of the blood used to determine complete blood count on a Hemavet automated cell counter (Sysmex KX-2, Sysmex Corporation, Kobe, Japan). The remaining blood was centrifuged at 4°C and the plasma was frozen at –80°C until analysis.

Kidneys and liver were immediately removed and weighed. The relative kidney weight and feeding efficiency were calculated using the following formulas:

$$\text{Relative kidney weight} = \left[ \frac{\text{weight of the kidney (g)}}{\text{body weight (g)}} \right] \times 100\%$$

$$\text{Feeding efficiency (\%)} = \left[ \frac{\text{body weight change (g)}}{\text{food intake (g)}} \right] \times 100\%$$

All animal experimentation procedures were conducted according to the Affidavit of Approval of Animal Use Protocol, Chung Shan Medical University Experimental Animal Center, Taichung, Taiwan (Approval No. 1439).

### Biochemical Blood Analysis

Urea, creatinine, and triglycerides were determined in the serum by colorimetry using a commercial kit (Randox Laboratories Ltd, Crumlin, UK), according to the manufacturer's protocols. Serum Na, K, and uric acid analyses were performed on automatic analyzer (ADVIA 1800, Siemens, Malvern, PA).

### Oxidative Stress and Antioxidant Enzymes in Kidney Homogenate Analysis

Thiobarbituric acid reactive substances resulted from acid-heating reaction and served as an index of lipid peroxidation according to a previously described method.<sup>30</sup> In brief, kidneys were homogenized in 10 volumes of PBS (pH 7.4). The homogenate supernatant was mixed with 40% trichloroacetic acid and 0.85% thiobarbituric acid, then heated in boiling water bath for 20 minutes. The malondialdehyde (MDA) concentrations were determined at 535 nm using 1,1,3,3-tetraethoxypropane as standard.<sup>30</sup> Tissue MDA was expressed as nmol/g protein. The protein concentration of kidney homogenates was determined by the Bradford method (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard. Catalase (CAT) activity was assayed by measuring the absorbance decrease of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) at 240 nm.<sup>31</sup> CAT activity was expressed as unit/mg protein. The glutathione (GSH) content of the kidney homogenate was measured using the method applied by Nazıroğlu et al<sup>20</sup> with some modifications. Briefly, the homogenate supernatant or the standard GSH solution was precipitated with 2% trichloroacetic acid (TCA) and then centrifuged at 1000g for 5 minutes. The reaction mixture contained 0.05 mL of supernatant, 20 mM PBS-EDTA buffer, 1.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB)-NaHCO<sub>3</sub>, 2 mM NADPH, and 1 unit mL<sup>-1</sup> GSH reductase. The solution was kept at room temperature for 10 minutes, and then read at 405 nm on the spectrophotometer (Ultrospec 2100 pro UV/Visible, Amersham Biosciences).

### Blood Cytokines

Cytokine and chemokine expression profiles in the sera of the 3 groups were evaluated simultaneously from a single well using the Bio-Plex System (Bio-Rad, Hercules, CA)

combined with Linco 14-Plex Rat Cytokine Detection Kit following the manufacturer's instructions. Briefly, the cytokine detection kit contains color-coded microspheres conjugated with a monoclonal antibody specific for a target protein. Antibody-coupled beads with the sample contain the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed with the addition of streptavidin phycoerythrin. The cytokine concentrations were expressed as the amount of cytokine in picograms per milliliter, and calculated by a standard curve. Regression analysis was performed to derive an equation to predict the concentrations of the unknown samples. This method was chosen because its assay principle is similar to that of a sandwich enzyme-linked immunosorbent assay method that provides a suitable rapid, sensitive analysis but requires much smaller sample volumes and is suitable for multiplexing. The measured cytokines were interleukin-1β (IL-1β), IL-2, IL-5, IL-7, IL-17, macrophage colony stimulating factor (M-CSF), growth-related oncogene (GRO/KC), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-3α and regulated upon activation, normal T-expressed, and secreted (RANTES).

### Histological Analysis

For the assessment of pathological changes, kidney was fixed in 10% formaldehyde solution and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H&E).

### Statistical Analysis

IBM SPSS Statistics 19 was used for all statistical analysis. All data are presented as mean ± standard deviation (SD). Statistical comparisons of the different treatment groups were carried out by one-way ANOVA followed by Tukey's test adjustments for multiple comparisons. *P* < .05 was considered statistically significant.

## Results

### D-Methionine Alleviates Weight Loss and Decreased Desire to Eat During the Cisplatin Treatment Period

In a previous study, guinea pigs treated with D-methionine combined with cisplatin lost less body weight than animals treated with cisplatin alone during short-term exposure.<sup>24</sup> To evaluate the long-term effect of D-methionine on cisplatin-induced body weight loss, cisplatin was administered ip once a week for 3 successive weeks. The body weight of

each rat was measured from day 1 to day 21 during the experimental period. On the 21th day, cisplatin administration for 3 successive weeks significantly decreased body weight by 31% when compared with control rats (Figure 1A). Weight loss was also observed in D-methionine-treated rats. The final body weight of D-methionine-treated group decreased 8% when compared with the control group.

Figure 1B shows that all animals treated with cisplatin had significantly decreased appetite after 3 weeks of consecutive administration. Before cisplatin injection, food intake in all rats was in the range of 27 to 28 g/day/rat. On day 2 after cisplatin treatment, food intake noticeably declined in all test groups except the control group. Food intake range was between 12 and 18 g/day/rat in cisplatin-alone group and cisplatin combined with D-methionine group (data not shown). The average food intakes in cisplatin-treated group and D-methionine co-treatment group decreased by 52% and 29%, respectively, compared with the control group at the end of the experimental period (Figure 1B).

In addition, feeding efficiency of the cisplatin-alone group was markedly lower than that of D-methionine co-treatment group (Figure 1C). The feeding efficiency gradually decreased from day 1 to day 18 in the cisplatin-alone group. The feeding efficiency is -120% (days 1-7), -224% (days 8-14), and -414% (days 15-19) when compared with the control group. The decreased feeding efficiency partially slowed after D-methionine administration.

### **Organ Weights, Relative Organ Weights, and Serum Renal Functional Parameters**

Cisplatin-treated rats showed significant decreases in liver weight and increases in kidney/body weight ratio when compared with the control group. The increase in kidney/body weight ratio indicated that the kidneys of cisplatin-treated rats were damaged.<sup>32</sup> D-methionine administration had obvious protective effects on liver and kidney/body weight ratio when compared with cisplatin-treated rats (Table 1). Serum sodium, blood urea nitrogen (BUN), and creatinine levels were increased in the cisplatin group when compared with the control group. Meanwhile, the concentrations of potassium and uric acid decreased in the cisplatin group when compared with the control group. D-methionine administration had no significant effect on serum sodium, BUN, creatinine, or uric acid levels, when compared with cisplatin-treated rats. Data also showed that the decrease in potassium concentration markedly recovered following D-methionine administration.

### **The Effects of Cisplatin and D-Methionine on Hematological Parameters**

Hematological parameters are presented in Table 2 and appear abnormal after 3 weeks of consecutive injection of

cisplatin in rats. White blood cell (WBC), mean corpuscular volume (MCV), and platelet values decreased, while mean corpuscular hemoglobin concentration (MCHC) increased in cisplatin-treated rats when compared with control rats. These abnormal changes were partially reversed with D-methionine administration. The recovery effect of oral D-methionine did not reach statistical significance for WBC, hemoglobin, or hematocrit.

### **Tissue Oxidative Stress/Antioxidant Status and Serum Triglyceride Levels**

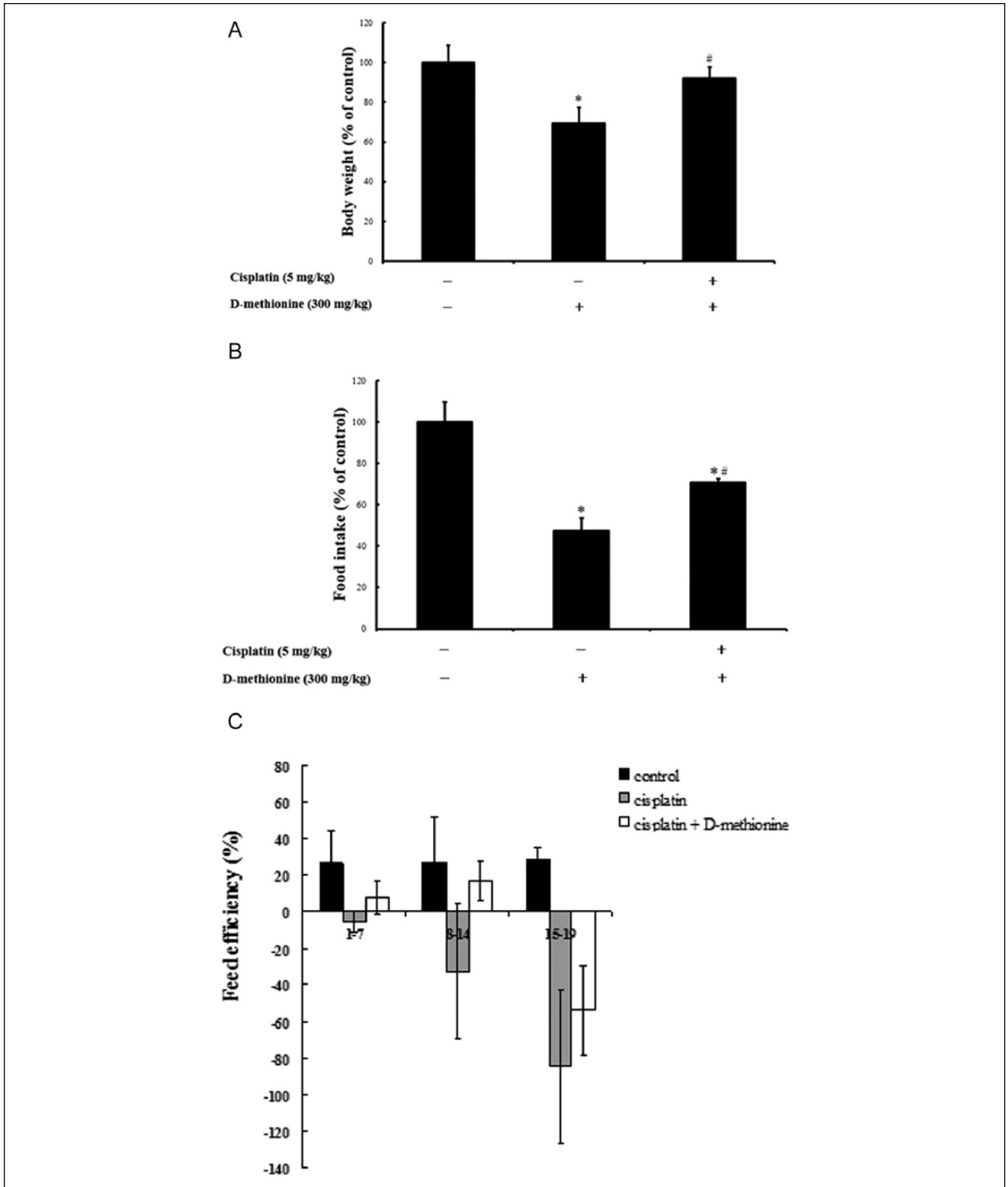
In the cisplatin group, GSH concentration and CAT activity were found to be depleted relative to the control group (Figure 2A and B). D-methionine administration elevated CAT activity, reflecting that D-methionine supports antioxidant activity and retards oxidative stress. D-methionine administration elevated GSH content relative to than of cisplatin-alone rats but the difference failed to reach statistical significance. The extent of lipid peroxidation, a marker of oxidative stress, was determined by the concentration of thiobarbituric acid reactive products (MDA). The level of kidney homogenate MDA was elevated in the cisplatin group when compared with control group (Figure 2C). We also observed that cisplatin promotes the levels of serum triglycerides as opposed to saline-treated rats but that D-methionine does not diminish the rise in triglyceride concentrations (Figure 2D).

### **ELISA Analysis**

Cisplatin significantly reduced GRO/KC level and raised MCP-1, MIP-1 $\alpha$ , and VEGF levels (Table 3). In cisplatin combined with 300 mg/kg D-methionine-treated animals, the production levels of IL-1 tended to be lower and the IL-2 and M-GSH levels tended to be higher when compared with cisplatin-treated rats. However, these levels did not differ between the cisplatin-alone group and the cisplatin combined with D-methionine group. Serum MCP-1, MIP-1 $\alpha$ , and VEGF levels were remarkably elevated in cisplatin-induced nephrotoxic rats when compared with the control group. Coadministration of D-methionine resulted in substantial decrease in VEGF and increase in GRO/KC. Based on these results, D-methionine has anti-inflammatory property.

### **Histopathological Examination**

Cisplatin mainly accumulated in kidney and caused renal lesions. Histopathologic examination was conducted to establish the degree of renal lesions. No markedly pathologic lesions were observed in control rats. Leukocyte infiltration, tubule vacuolization, tubular expansion, and swelling were found in cisplatin-treated



**Figure 1.** Effect of D-methionine on body weight (A), food intake (B), and feeding efficiency (C) after cisplatin injection. All animals were sacrificed on the 21st day of the experiment and body weight and food intake were recorded.

\*Indicates statistical significance when compared with the control group ( $P < .05$ ).

#Indicates statistical significance when compared with the cisplatin group ( $P < .05$ ).

**Table 1.** Effects of D-Methionine on Cisplatin-Induced Changes in Organ Weights, Kidney to Body Weight Ratios, and Blood Biochemical Parameters<sup>a</sup>.

	Control	Cisplatin	Cisplatin + D-Methionine
Liver (g)	10.3 ± 1.1	7.6 ± 1.1*	10.3 ± 0.8c <sup>#</sup>
Kidney (g)	1.3 ± 0.1	1.5 ± 0.4	1.5 ± 0.2
Kidney/BW ratio (%)	0.74 ± 0.02	1.28 ± 0.14*	0.96 ± 0.09 <sup>#</sup>
Na (mEq/L)	142.6 ± 1.3	144.7 ± 1.8*	145.0 ± 1.5*
K (mEq/L)	8.2 ± 1.2	6.5 ± 0.4*	8.0 ± 1.3 <sup>#</sup>
BUN (mg/dL)	21.0 ± 2.4	57.4 ± 23.3*	42.0 ± 22.7
Creatinine (mg/dL)	0.39 ± 0.07	0.67 ± 0.20*	0.68 ± 0.26*
Uric acid (mg/dL)	5.0 ± 2.2	2.7 ± 0.4*	4.7 ± 0.6

Abbreviations: BW, body weight; BUN, blood urea nitrogen.

Values are presented as mean ± SD of 7 rats.

\*Indicates statistical significance when compared with the control group ( $P < .05$ ).

<sup>#</sup>Indicates statistical significance when compared with the cisplatin group ( $P < .05$ ).

**Table 2.** Hematological Parameters in Control and Experimental Groups.

	Control	Cisplatin	Cisplatin + D-Methionine
WBC ( $10^3/\mu\text{L}$ )	10.4 ± 2.1	4.0 ± 1.2*	6.7 ± 1.3*
RBC ( $10^6/\mu\text{L}$ )	8.3 ± 0.2	7.9 ± 1.1	8.1 ± 0.7
Hemoglobin (g/dL)	15.7 ± 0.7	14.7 ± 1.7	16.1 ± 1.5
Hematocrit (%)	50.2 ± 2.1	45.5 ± 6.0	50.6 ± 3.6
MCV (fL)	60.7 ± 1.5	57.9 ± 1.0*	62.3 ± 1.7 <sup>#</sup>
MCH (pg)	19.0 ± 0.4	19.7 ± 0.7	19.7 ± 0.4
MCHC (g/dL)	31.3 ± 0.4	34.0 ± 1.3*	31.7 ± 0.7 <sup>#</sup>
Platelet ( $10^3/\mu\text{L}$ )	902.0 ± 109.8	124.8 ± 99.3*	806.8 ± 168.3 <sup>#</sup>

Abbreviations: WBC, white blood cell; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration.

Values are presented as mean ± SD of 7 rats.

\*Indicates statistical significance when compared with the control group ( $P < .05$ ).

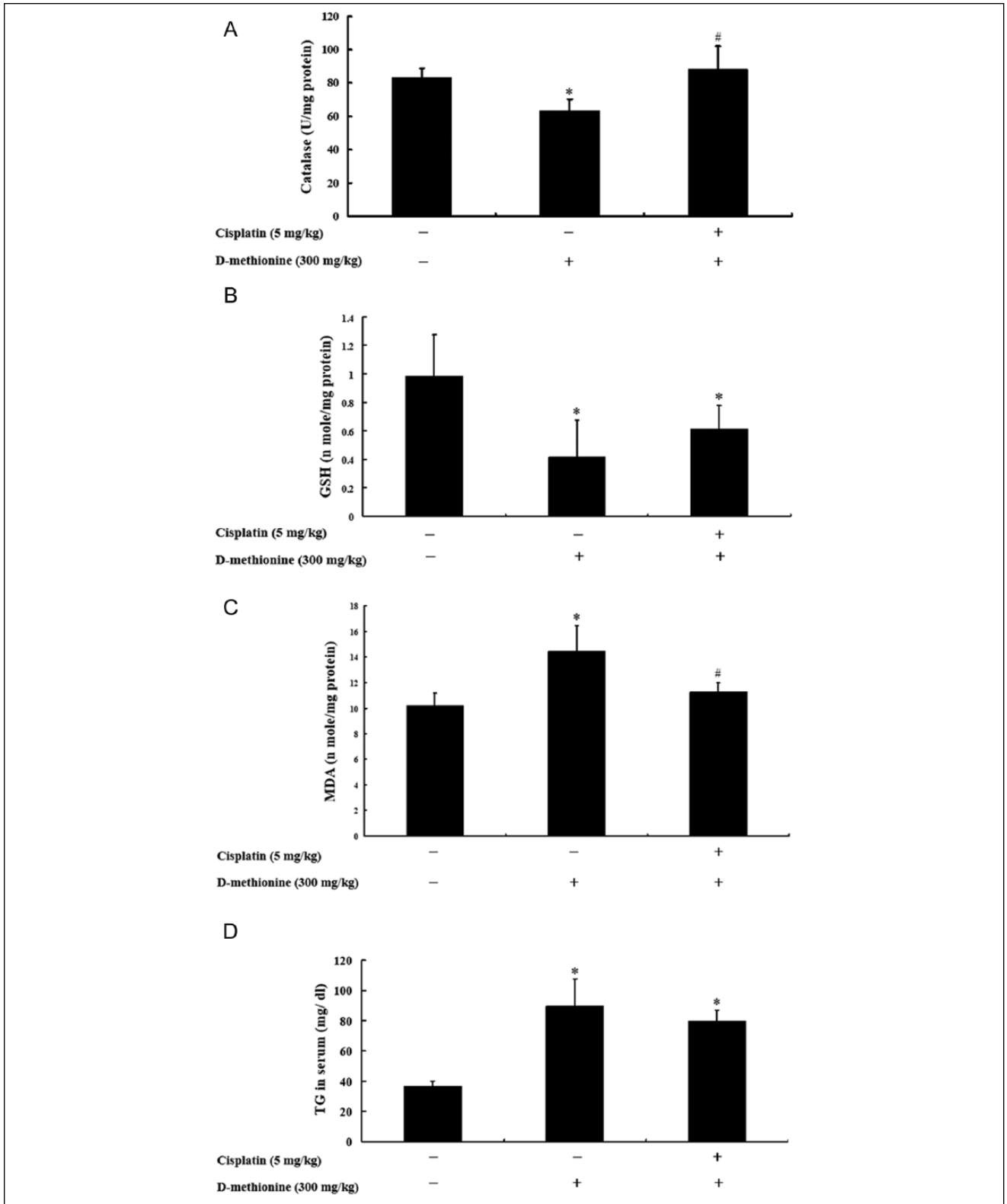
<sup>#</sup>Indicates statistical significance when compared with the cisplatin group ( $P < .05$ ).

rats (Figure 3). Severity of lesions was attenuated by D-methionine administration.

## Discussion

Cisplatin-induced nephrotoxicity is closely associated with oxidative stress and inflammation.<sup>33</sup> Using antioxidant compounds to mitigate cisplatin-induced oxidative stress and various adverse effects has been recommended.<sup>34</sup> It has been reported that D-methionine, a sulfur-containing amino acid, reduces the ototoxicity and nephrotoxicity of cisplatin without decreasing antitumor action.<sup>12,35</sup> MRX-1024, a high concentration (200 mg/mL) bioavailable suspension formulation of D-methionine, has been shown to be appropriate for head and neck cancer patients receiving concurrent radiation and cisplatin.<sup>36</sup> In the present study, we demonstrated that D-methionine ameliorates cisplatin-induced anorexia by improvement of food intake, leading to weight gain (Figure 2A and B). Meanwhile, D-methionine lessened kidney damage caused by cisplatin, which was mediated by regulation of antioxidant/oxidative stress.

Food intake decrease and weight loss are the 2 most common and serious health problems in patients with cancer undergoing chemotherapy, especially in those taking cisplatin.<sup>7</sup> Research has indicated that the administration of cisplatin significantly reduces food intake on the first day after treatment, reaching a nadir at 2 days, and progressively recovering thereafter.<sup>37</sup> It is well known that cisplatin has highly emetic effect. Cabezos et al<sup>38</sup> reported that cisplatin dose-dependently induced both gastric stasis and stomach distension. Also, gastric distension is associated with food retention. Gastric motility was impaired in cisplatin-treated rats.<sup>39</sup> Cisplatin led to a decrease in locomotor activity and gastric motility.<sup>37,39</sup> Malaise (physical activity reduced), food intake reduction, weight loss, stomach distension (data not shown), and renal damage caused by cisplatin were observed at the end of this study. We also found that the toxic effects of cisplatin resulting in appetite and weight losses, as well as diminished feeding efficiency, are enhanced by accumulating doses and treatment times (Figure 1). Our results are in agreement with a previous



**Figure 2.** Effects of D-methionine on catalase activity (A), GSH concentration (B), lipid peroxidation (C) in the kidney, and serum triglyceride concentration (D) in cisplatin-induced nephrotoxicity. Values are presented as mean  $\pm$  SD.

\*Indicates statistical significance when compared with the control group ( $P < .05$ ).

#Indicates statistical significance when compared with the cisplatin group ( $P < .05$ ).

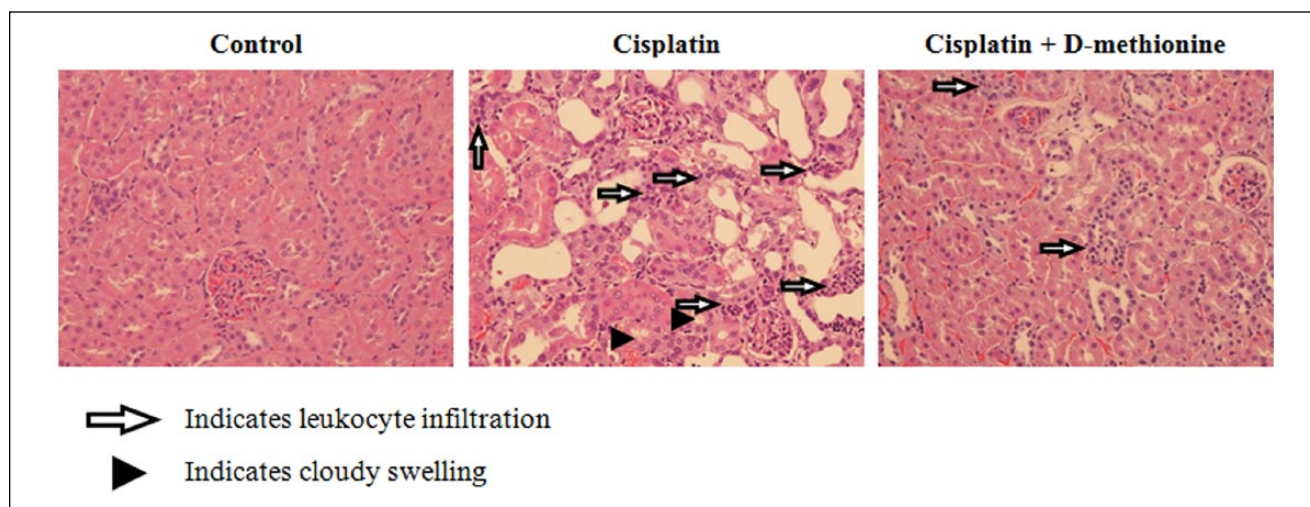
**Table 3.** Effects of D-Methionine on Cytokines in Cisplatin-Induced Toxicity.

	Control	Cisplatin	Cisplatin + D-Methionine
GRO/KC	100.4 ± 26.1	31.6 ± 22.9*	97.7 ± 29.0 <sup>#</sup>
IL-1β	8.0 ± 1.2	13.5 ± 8.7	8.6 ± 0.6
IL-2	98.6 ± 24.8	64.1 ± 13.8	92.5 ± 37.2
IL-5	72.5 ± 14.4	74.4 ± 10.8	90.5 ± 18.4
IL-7	65.4 ± 23.1	40.4 ± 29.2	79.8 ± 51.3
IL-17	16.7 ± 6.8	8.9 ± 2.1	9.0 ± 5.5
MCP-1	1217.9 ± 198.6	2474.1 ± 664.3*	1661.2 ± 563.3
M-CSF	83.6 ± 12.3	102.1 ± 8.5	80.8 ± 14.5
MIP-1α	22.8 ± 2.9	50.5 ± 12.3*	40.2 ± 14.4
MIP-3α	25.2 ± 3.4	25.5 ± 2.4	31.7 ± 5.8
RANTES	367.7 ± 56.9	396.8 ± 209.8	628.3 ± 192.8
VEGF	8.6 ± 1.3	30.9 ± 16.6*	10.2 ± 6.1 <sup>#</sup>

Values are presented as mean ± SD of 7 rats.

\*Indicates statistical significance when compared with the control group ( $P < .05$ ).

<sup>#</sup>Indicates statistical significance when compared with the cisplatin group ( $P < .05$ ).



**Figure 3.** Representative photographs from histopathological analyses: kidney after cisplatin and D-methionine administration, using H&E staining. Alterations were observed in cisplatin and cisplatin plus D-methionine groups. In cisplatin-treated rats, there was clear leukocyte infiltration, tubule vacuolization, tubular expansion, and cloudy swelling in kidney tissues. However, following administration of D-methionine, there was moderate degree of lesions of cisplatin-induced toxicity.

report by Garcia et al,<sup>7</sup> who observed cisplatin-induced appetite, body weight and feeding efficiency decreases.

To our knowledge, this is the first report of D-methionine-mediated prevention of cisplatin-induced anorexia. In this study, D-methionine-mediated weight gain was clearly visible after the first cisplatin injection. In contrast, weight loss caused by cisplatin became progressively more severe with accumulated dosage of cisplatin. It is interesting to note that D-methionine not only improved food intake but also increased body weight after 3 consecutive doses of cisplatin. These results implied that D-methionine led to better food intake and prevented body weight loss under cisplatin intervention. This was consistent with the findings of

Campbell et al<sup>22</sup> of constant effect of oral D-methionine on weight gain in cisplatin-induced ototoxic rat model over a short period of time. From these results, co-treatment with D-methionine has anti-anorexic effect.

Based on food intake and body weight change, feeding efficiency was calculated. We monitored the total food intake during days 1 to 7, 8 to 14, and 15 to 19, as there were significant differences in food consumption between the groups in this period. Clearly, cisplatin injection resulted in a marked attenuation of food efficiency, which was significantly different from control and D-methionine-treated rats (Figure 1C). According to Garcia et al,<sup>7</sup> feed efficiency was calculated to understand the contribution of caloric intake



on the weight/mass changes induced by cisplatin. Results of feeding efficiency revealed that D-methionine prevents reduced caloric intake that is caused by cisplatin. Previous studies have mostly focused on the role of D-methionine in alleviating cisplatin-induced ototoxicity<sup>12,22,24</sup> rather than on food intake and weight loss caused by cisplatin. Moreover, the model of cisplatin administration in those studies was short term and high dose. The data from our model of repeated administration of low-dose cisplatin revealed that coadministration of D-methionine has orexigenic effects (ie, increases appetite) on cisplatin-induced anorexia.

Cisplatin-induced anorexia is caused by complex multifactorial processes that have yet to be fully elucidated. Gastrointestinal tract disorders including vomiting, nausea, stomach distension, and gastric stasis may result in decreased food intake.<sup>37</sup> The symptoms of gastrointestinal dysfunction caused by cisplatin are associated with the activation of abdominal vagal afferents and the release of endogenous satiety hormones.<sup>37,40</sup> In situ hybridization histochemistry and reverse transcriptase polymerase chain reaction analyses have shown that many feeding-regulating peptides in the hypothalamus are involved in cisplatin-induced anorexia.<sup>6,37</sup> Cisplatin-induced anorexia has also been demonstrated to be mediated by decreases in plasma ghrelin level and serotonin (5-HT) secretion from enterochromaffin cells.<sup>41</sup> This long-term cisplatin treatment study indicated that both reduction in food ingestion and reduced capacity of the body to efficiently use the food ingested lead to weight loss.<sup>42</sup> Therefore, anorexia is thought to be the critical cause of body weight loss. To date, the exact mechanisms of protective effects of D-methionine on cisplatin-related gastrointestinal dysfunction are not clear. It has been reported that platinum-containing hydrolysis products were generated in the bloodstream after the intravenous injection of cisplatin. These products were thought to be more toxic than the parent drug.<sup>43</sup> Interestingly, the formation of some cisplatin–D-methionine species may mitigate the toxic side effects of cisplatin by inactivating these hydrolysis products in vivo with co-administering D-methionine (at the molar ratio of 20:1, D-methionine–cisplatin).<sup>44</sup> It is well known that methionine is converted to cysteine, taurine, and glutathione via transsulfuration. As reviewed by Wu, glutathione is an important intracellular antioxidant and redox potential regulator and is able to enhance antioxidant activity. Meanwhile, glutathione exerts a beneficial effect on cytoprotection.<sup>45</sup> In addition, cysteine and glutathione may regulate epithelial cell proliferation via modulation of redox status.<sup>46</sup>

In fact, L-methionine has also been proven to effectively alleviate cisplatin-induced toxic side effects but D-methionine exhibits a longer lifetime in the bloodstream and its use is therefore advantageous.<sup>44</sup> Methionine likely plays a critical role in intestinal cell function

and antioxidant status.<sup>46</sup> Recently, D-methionine has been considered a good chemoprotectant against radiation and/or chemotherapy-induced side effects.<sup>22,24,26</sup> Hence, further research could be carried out to determine the potential use of D-methionine for clinical use. The mechanism of D-methionine against cisplatin-induced anorexia requires further study to help establish the mechanisms of D-methionine in improving appetite and inducing weight gain.

A recent clinical trial has shown that bone marrow depression frequently occurs following the administration of cytostatic agents such as cisplatin.<sup>47</sup> Chemotherapy-induced hematological toxicities such as neutropenia and anemia can increase the likelihood of life-threatening infections. Chemotherapy-related hematopoietic suppression has been observed from hemoglobin, platelet, and lymphocyte data after cisplatin administration.<sup>48</sup> In the present study, hemoglobin, hematocrit, and red blood cell values did not significantly differ between cisplatin and control rats. However, major reductions in WBC and platelet counts, as well as notable increases in MCHC, were recorded in cisplatin-treated rats when compared with control rats. After treatment with D-methionine, hematological parameters markedly improved (Table 2). Although WBC counts tended to increase following administration of D-methionine, this increase did not reach statistical significance. The current study demonstrated that oral administration of D-methionine attenuates hematological toxicity induced by cisplatin.

Cisplatin-induced nephrotoxicity includes tubular damage and tubular dysfunction with sodium, potassium, and magnesium wasting. Decreased levels of serum potassium are due to decreased glomerular filtration.<sup>49</sup> Decreases in potassium levels and increases in kidney/body weight ratio were recovered following D-methionine administration (Table 1). This is in agreement with previous reports that cisplatin induces significant elevation in relative kidney weight, an indicator of kidney damage.<sup>50</sup> It is well recognized that cisplatin is preferentially taken up by the proximal tubule with accumulation in renal tubular cells, and consequently leads to renal dysfunction and injury. Histopathological changes in the kidney and biochemical parameters of blood (BUN and creatinine levels) are both considered detection markers of kidney injury. Common histopathological characteristics of renal toxicity induced by cisplatin include tubular dilation, tubular epithelial degeneration, and proteinous casts, among others.<sup>51</sup> Cisplatin-induced acute kidney injury is associated with neutrophil infiltration in the kidney.<sup>52</sup> In the present study, extensive lesions in the kidney tissues of cisplatin-treated rats after the third injection are shown in Figure 3, with clear leukocyte infiltration, tubule vacuolization, tubular expansion, and cloudy swelling. The increases in kidney weight/body weight ratio, blood creatinine, and BUN level,

and disturbances in electrolyte homeostasis prove that the administration of cisplatin causes renal dysfunction. Despite the creatinine and BUN level increases (Table 1), the markedly pathologic lesions and the ratio increase in kidney weight/body weight were alleviated by D-methionine supplements. These results were in agreement with previous studies and suggested that D-methionine is partially able to ameliorate cisplatin-induced nephrotoxicity in a chronic toxicity model.<sup>53</sup>

Although the pathological mechanisms of nephrotoxicity caused by cisplatin are unknown, oxidative and inflammatory stresses have been widely researched. Several inflammatory cytokines and chemokines have been shown to be elevated in rats with cisplatin-induced nephrotoxicity.<sup>54</sup> Cisplatin can induce production of renal TGF- $\beta$ R1, TGF- $\beta$ 1, TNF- $\alpha$ , IL-1 $\beta$  and decrease antioxidant enzyme activity.<sup>55</sup> In the present study, cisplatin increased MCP-1, M-CSF, and VEGF levels in blood. IL-1 levels also increased in rats administered cisplatin. However, this increase was only slightly different when compared with control values. We also found that D-methionine administration does not reduce cisplatin-induced increases in MCP-1 and M-CSF levels. GRO/KC, also known as CXCL1, is a potent neutrophil chemoattractant. It has been previously demonstrated that CXCL1 increases in the kidney in cisplatin-induced acute kidney injury.<sup>54</sup> Particularly, our data indicated decreased GRO/KC levels in the blood of cisplatin-treated rats on day 21 when compared with control rats ( $P < .05$ ). Administration of oral D-methionine significantly increased GRO/KC level. Inconsistent with a previous study,<sup>54</sup> cisplatin lowered blood GRO/KC. We speculate that this discrepancy was due to our model of 3-week chronic experimental period and low dose (5 mg/kg), which differed from that of the previous study.<sup>54</sup> In addition, rats were sacrificed on day 6 instead of day 3 after cisplatin administration. A plasma cisplatin concentration-time curve study has demonstrated that the concentration of plasma reaches its peak at approximately 18 minutes after cisplatin injection with linear rapid decrease during the first 6 hours and elimination of cisplatin from blood within a 72-hour period.<sup>56</sup> These findings suggest that GRO/KC is worthy of further investigation for its role in cisplatin-induced toxicity. A previous immunohistochemistry assay has indicated that VEGF, which leads to vascular inflammation, is expressed in rat kidneys following a single dose of cisplatin (4 mg/kg) once a week for 4 weeks.<sup>49</sup> Our data revealed that cisplatin causes overproduction of VEGF and D-methionine, effectively decreasing VEGF levels in blood.

As mentioned above, antioxidants are considered effective for ameliorating cisplatin-induced nephrotoxicity. Cisplatin contributes to the significant increase in renal MDA, a marker of lipid peroxidation, and decrease in CAT activity.<sup>23,55</sup> In addition, cisplatin decreases

glutathione levels in kidney.<sup>57</sup> Hence, an imbalance in the antioxidant defense system impairs renal function. The present results demonstrated that D-methionine improves cisplatin-induced oxidative stress events as indicated by suppression of MDA levels, slightly elevated GSH concentration, and catalase activity in kidney homogenate (Figure 2). Methionine maintains GSH concentration in kidney cortices.<sup>58</sup> In addition, D-methionine may act as a sulfur-containing nucleophile and has metal chelating property for scavenging free radicals.<sup>12,59</sup> Moreover, previous studies have demonstrated the antioxidative properties of D-methionine in cisplatin-induced adverse effects.<sup>24,25</sup> Together, these results suggest that D-methionine has potential protective effect against inflammatory and oxidative stresses caused by the anti-cancer drug cisplatin.

Increasing serum triglyceride levels have been observed in cisplatin-treated animals with a disturbance in lipid metabolism.<sup>60,61</sup> Cisplatin impairs fatty acid oxidation, which leads to increased triglyceride accumulation in renal tubules.<sup>61</sup> Our study showed that increases in the levels of triglyceride in serum following cisplatin injection are not diminished by D-methionine intake. This suggested that D-methionine is unable to prevent cisplatin-induced disturbance in lipid metabolism. Some reports have shown an increase in circulating triglycerides on the fourth day of cisplatin treatment. However, in one study, no substantial differences in circulating triglycerides were observed in cisplatin-treated animals on day 44.<sup>7</sup> Another study showed that when cisplatin is injected on the seventh day it results in a significant decrease in triglyceride serum levels.<sup>23</sup> We speculated that the effects of cisplatin on triglyceride changes are dose and time dependent.<sup>49</sup>

In addition to D-methionine, some low-molecular-weight compounds have also been shown to be effective at reducing the side effects of cisplatin, such as sodium thiosulfate,<sup>62,63</sup> N-acetyl-L-cysteine,<sup>64</sup> and others chemoprotective agents.<sup>65</sup> Furthermore, the fortification of plasma with pure plasma proteins has been shown to offer some protection against the toxic effects of cisplatin.<sup>43</sup>

In conclusion, D-methionine has been recommended for decreasing the ototoxicity of cisplatin. In this study, we found that the chemopreventive efficacy of D-methionine includes improved appetite, weight gain, and attenuated nephrotoxicity through elevation of antioxidative activities in a chronic repeated dosing model. Our results support the use of D-methionine as a chemoprotectant during chemotherapy.

#### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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