Title

Antioxidant effect of ascorbic acid against cisplatin-induced nephrotoxicity and P-glycoprotein expression in rats

Keisuke Okamoto [a, 1], Fumi Kitaichi [a, 1], Yoshitaka Saito [b, *], Hinata Ueda [a], Katsuya Narumi [a], Ayako Furugen [a], Masaki Kobayashi [a, c, **]

[a] Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo, 060-0812, Japan

[b] Department of Pharmacy, Hokkaido University Hospital, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo, 060-8648, Japan

[c] Education Research Center for Clinical Pharmacy, Faculty of Pharmaceutical Sciences, Hokkaido University; Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan

[1] These authors contributed equally as co-first authors.

*, **, To whom correspondence should be addressed.

Corresponding Author: Yoshitaka Saito & Masaki Kobayashi

Department of Pharmacy, Hokkaido University Hospital, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo, 060-8648, Japan. E-mail: yossypma@med.hokudai.ac.jp (Yoshitaka Saito)

Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo, 060-0812, Japan. E-mail: masaki@pharm.hokudai.ac.jp (Masaki Kobayashi)

Abstract

Cisplatin (CDDP) is a highly potent anticancer drug that is widely used in the treatment of several cancers. CDDP-induced nephrotoxicity (CIN) is one of the most significant adverse effects, and oxidative stress is thought to be one of the mechanisms underlying CIN. Although there are some studies available on the variability in transporter expression in the kidney after a single CDDP dose, none have reported the change in renal transporter expression after multiple CDDP dose administrations. P-glycoprotein (P-gp), a transporter, is reported to be induced by oxidative stress. Ascorbic acid is a vitamin with antioxidant potential and therefore, may regulate the expression of P-gp transporter and affect CIN. In the present study, our aim was to assess the variability in expression of several renal transporters after multiple CDDP dose administrations and the antioxidant effect of ascorbic acid against transporter expression and CIN. Multiple doses of CDDP affected markers of kidney injury and antioxidants in the kidneys. Also, the expression of P-gp, breast cancer resistance protein, and multidrug resistance-associated protein 4 was upregulated by CDDP. Using a normal kidney cell line, we demonstrated that ascorbic acid attenuated CDDP-induced cytotoxicity due to its high superoxide scavenging ability. CDDP and ascorbic acid were injected into rats once a week for three weeks, and it was observed that co-administration of ascorbic acid attenuated CIN and regulated antioxidant marker. In addition, ascorbic acid reduced P-gp expression, which was upregulated by CDDP. In conclusion,

ascorbic acid may attenuate CIN and reverse P-gp-mediated changes in drug pharmacokinetics.

Keywords

Cisplatin; Ascorbic acid; Nephrotoxicity; P-glycoprotein; Antioxidant; Transporter

1. Introduction

Cisplatin (CDDP) is a highly potent anticancer drug that is widely used in several cancer treatments (Rosenberg et al., 1969). CDDP is an alkylating agent that exerts anticancer effect by binding to DNA, following which, defective DNA templates cause an arrest of DNA synthesis and replication (Pabla and Dong, 2008). In addition, production of reactive oxygen species (ROS) has been reported to be involved in the anticancer effect of CDDP (Casares et al., 2012).

CDDP-induced nephrotoxicity (CIN) is one of the most significant adverse effects associated with CDDP treatment (Prestayko et al., 1979). CIN is dose-dependent and cumulative, and occurs in approximately 30% of patients who receive CDDP (Miller et al., 2010). The mechanism of CIN involves DNA damage, oxidative stress, and other processes (Kawai et al., 2006; Park et al., 2002). Although hydration has been used clinically to prevent CIN, it has not been reported to attenuate CIN to an adequate extent (de Jongh et al., 2003).

We have reported that co-administration of magnesium attenuates CIN via the regulation of transporters including organic cation transporter 2 (Oct2), and multidrug and toxin extrusion 1 (Mate1) with a single dose and copper transporter 1 (Ctr1) with multiple-dose administrations (Saito et al., 2017a; Saito et al., 2017b). In addition, not only the transporter expression level, but also platinum accumulation in the kidney were different with the single and multiple doses of CDDP. Although some studies have evaluated the variability in transporter expression in the kidney using a single CDDP dose (Morisaki et al., 2008; Solanki et al., 2014), there are no studies available that report changes in renal transporter expression following multiple CDDP dose administrations. The variability in transporter expression due to multiple doses is important because CDDP is usually administered to patients with cancer via multiple doses in clinical settings. Moreover, evaluation of the expression of transporters that do not recognize CDDP as a substrate is also meaningful, considering the pharmacokinetics of other concomitant drugs.

Ascorbic acid is a vitamin that has been suggested to have antioxidant potential (Padayatty and Levine, 2016). It has been reported that the expression of P-glycoprotein (P-gp), a transporter expressed in the kidney, is induced by ROS (Ziemann et al., 1999). Since P-gp recognizes and enhances the excretion of many drugs (Yee et al., 2018), the antioxidant effect of ascorbic acid is considered to suppress the induction of P-gp expression, resulting in improved pharmacokinetics of these drugs. Although the antioxidant effect of ascorbic acid

has been reported to reduce CIN (Abdel-Daim et al., 2019), to our knowledge, no study has evaluated the effects of ascorbic acid on multiple low-dose administrations of CDDP. Cisplatin is an anticancer drug that is administered multiple times in clinical practice, and it accumulates in the kidney; therefore, evaluation in effects of repeated administrations of cisplatin is important. The aim of the present study is to assess the variability in expression of several renal transporters caused by multiple CDDP administrations, and the antioxidant effect of ascorbic acid against transporter expression and CIN.

2. Materials and Methods

2.1. Chemicals

CDDP and L-ascorbic acid were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). The primary anti-Oct1 (AI11904; 61 kDa) antibody was from Abgent (San Diego, CA, USA); anti-Oct2 (H-62; 63 kDa) and anti-Mate1 (S-14; 62 kDa) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA); anti-organic anion transporter 1 (Oat1; ab131087; 62 kDa), anti-breast cancer resistance protein (Bcrp; ab3380; 70 kDa), anti-multidrug resistance-associated protein 2 (Mrp2; ab15603; 185 kDa), and anti-Mrp4 (ab77184; 170 kDa) antibodies were from Abcam (Cambridge, UK); anti-Oat3 (KAL-KE035; 62 kDa) antibody was from COSMO BIO (Tokyo, Japan); anti-P-gp (C219; 140 kDa) antibody was from GeneTex (Irvine, CA, USA); and anti-Actin (MAB1501; 42 kDa) antibody

was from Merck Millipore (Billerica, MA, USA). All other chemicals and reagents were commercially available and were of the highest purity.

2.2. Animals and experimental design

Male Wistar rats (7 weeks old) were procured from CLEA Japan (Tokyo, Japan). All rats were housed in an animal maintenance facility room with controlled temperature (23°C) and moisture (60 ± 10%) conditions and a 12 h light–dark cycle. All rats were given free access to food (demineralized diet pellets) and water. All animal experiments were conducted in accordance with the guidelines for the Care and Use of Laboratory Animals of Hokkaido University, and all experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the "Guide for the Care and Use of Laboratory Animals" (approval number: 17-0005).

CIN rat model of multiple CDDP dose administrations was established according to a previously published method (Saito et al., 2017a). Rats were treated with either CDDP (2.5 mg/kg, intraperitoneal (i.p.), once a week for three weeks) or saline. Ascorbic acid (100 mg/kg, once a week for three weeks, i.p.) or saline was co-administered, 1 h before CDDP administration. On day 22, blood samples were collected from the tail vein. Further, the rats were anesthetized using sevoflurane and euthanized, and the kidneys, liver, jejunum, and ileum were immediately excised. All collected tissues were washed in saline and stored at −80°C until further analysis.

2.3. Cell culture

The epithelioid clone of normal rat kidney cells (NRK-52E cells; JCRB Cell Bank, Osaka, Japan) was cultured in Dulbecco's Modified Eagle's medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma Aldrich) in a humidified atmosphere of 5% CO_2 at 37°C.

2.4. Measurement of serum creatinine level

Blood samples were centrifuged at 1,200 × g for 20 min at 4°C. The serum creatinine level in the supernatant was measured using LabAssay^{TM} Creatinine (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) in accordance with the manufacturer's protocol.

2.5. mRNA expression determination

Total RNA was extracted using an ISOGEN II kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Total RNA was used to prepare complementary DNA by reverse transcription using ReverTra Ace (Toyobo, Osaka, Japan). The mRNA levels were measured by quantitative polymerase chain reaction (qPCR) that was performed using a KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA, USA) and Mx3000p (Agilent Technologies, Santa Clara, CA, USA). Table 1 lists the primer sequences used for qPCR. The qPCR thermocycling protocol was: 40 cycles of denaturation at 95°C for 5 s; annealing at 58°C for 20 s; and extension at 72°C for 30 s. Standard curves were constructed for each target and housekeeping gene. The relative amounts of the target gene and the

housekeeping gene were calculated using the software, based on their threshold cycles.

2.6. Western blotting

The rat kidney cortex tissue was sliced and a lysate prepared by homogenization at 4°C in a lysis buffer containing 1.0% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 4.5 M urea, and cOmplet^{TM} Mini EDTA-free Protease Inhibitor Cocktail (Sigma Aldrich). All the samples were sonicated for 15 min and centrifuged at 12,000 × g for 15 min at 4°C. The protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). All lysates were added to a loading buffer containing 0.1 M tris-hydrochloride, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.004% bromophenol blue. Then, the samples were separated using SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Tokyo, Japan) by semidry electroblotting at 15 V for 90 min. The membranes were blocked with phosphate-buffered saline containing 0.05% Tween 20 and 1% non-fat dry milk for 1 h at room temperature. Next, the membranes were incubated overnight at 4°C with primary antibodies against Oct1 (1:400), Oct2 (1:200), Oat1 (1:1,000), Oat3 (1:100), Mate1 (1:100), Bcrp (1:250), Mrp2 (1:50), Mrp4 (1:200), P-gp (1:50), and Actin (1:500), and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies. After washing, the bound antibodies were detected using ECL Prime and Image Quant LAS 4000 (GE Healthcare UK Ltd., Buckinghamshire, UK). Band intensities were analyzed using ImageJ analysis software (NIH, Bethesda, MD, USA).

2.7. Cell viability assay

The cells were seeded separately in 96-well plates at a density of 5,000 cells/well in the culture medium. After 24 h, the cells were treated with CDDP and/or ascorbic acid (containing 0.1% dimethyl sulfoxide (DMSO)) for 48 h, and cell viability was measured by the MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in accordance with the manufacturer's instructions. The absorbance of the resulting reaction solution was measured using a test wavelength at 540 nm for the samples and a reference wavelength at 690 nm. The concentration of 50% inhibition for cell viability (IC_{50}) was calculated using SigmaPlot 14 (HULINKS Inc., Tokyo, Japan).

2.8. Superoxide scavenging assay

Superoxide scavenging ability was measured using 2-methyl-6-p-methoxyphenylethynyl-imidazopyrazinone (MPEC; ATTO, Tokyo, Japan) according to the manufacturer's protocol. Each reaction solution was placed in a 384-well white plate for chemiluminescence measurement.

2.9. Statistical analysis

Statistical analyses of data were performed using the unpaired Student's *t*-test or one-way ANOVA followed by the Tukey's post-hoc test. Data were analyzed using SigmaPlot 14; $P < 0.05$ indicated statistical significance.

3. Results

3.1. Effects of multiple CDDP dose administrations on oxidative stress

The effects of multiple CDDP dose administrations on the kidneys were evaluated, and mRNA level of tissue inhibitor of metalloproteinase 1 (Timp-1), a kidney injury marker, was observed to be increased in the kidney (Fig. 1A). In addition, we demonstrated that serum creatinine level and mRNA level of kidney injury marker 1 (Kim-1), which are also kidney injury markers, were consistent with our previous report (data not shown) (Saito et al., 2017a). Moreover, evaluation of antioxidant markers in the kidney, liver, jejunum, and ileum revealed that superoxide dismutase 1 (Sod1) mRNA level was decreased in the kidney and liver (Fig. 1B), while heme oxygenase 1 (Ho-1) mRNA level was increased only in the kidney (Fig. 1C). Therefore, the results suggest that multiple CDDP injections induce nephrotoxicity and oxidative stress.

3.2. Change of kidney transporter expressions by multiple CDDP dose administrations

We have reported that the expression of Ctr1 was unaffected by multiple CDDP dose administrations (Saito et al., 2017a), and therefore, evaluated the variability in the expression of other renal transporters. With respect to the uptake transporters, none of them was affected by multiple CDDP dose administrations (Fig. 2A and Fig. 2B). On the other hand, the expression of efflux transporters such as Bcrp, Mrp4, and P-gp was increased by multiple CDDP injections (Fig. 2C and Fig. 2D).

3.3. Effect of antioxidant activity on CDDP–induced cytotoxicity

Since CIN involves ROS production (Kawai et al., 2006), and P-gp expression is increased by ROS production (Ziemann et al., 1999), we focused on antioxidant activity of ascorbic acid. Evaluation using NRK-52E cells, which were used to search for drugs associated with CDDP-induced cytotoxicity in our previously report (Okamoto et al., 2020), revealed that the IC_{50} value of CDDP with ascorbic acid was higher than that without ascorbic acid (with ascorbic acid: $IC_{50} = 9.3 \pm 0.1$ μM, without ascorbic acid: $IC_{50} = 5.9 \pm 0.1$ μM; Fig. 3A). Moreover, we evaluated the superoxide scavenging potential of ascorbic acid and observed that it was higher than that of N-acetylcysteine, which was a positive control (Fig. 3B). Therefore, ascorbic acid might attenuate CDDP–induced cytotoxicity by reducing oxidative stress.

3.4. Effects of ascorbic acid on CIN and P-gp expression

To evaluate the effect of ascorbic acid on CIN, CDDP and ascorbic acid were co-administered to rats once a week for three weeks. Although there was no statistically significant difference, the co-administration of ascorbic acid did not affect weight loss induced by CDDP (Fig. 4A). However, ascorbic acid significantly decreased serum creatinine level compared to CDDP (Fig. 4B). In addition, co-administration of ascorbic acid prevented CDDP-induced decrease in Sod1 mRNA levels (Fig. 4C). Moreover, ascorbic acid significantly reversed the increase in P-gp expression induced by CDDP (Fig. 4D and Fig.

4E). These results suggested that ascorbic acid attenuated CIN and decreased the expression of P-gp.

4. Discussion

A CIN rat model was used in this study; the rats were repeatedly administered low doses of CDDP and the resulting change in transporter expression level was measured, which might reflect clinical manifestations, as CDDP is administered to patients with cancer via multiple doses in clinical settings. Although there are some studies on the change in renal transporter expression level after a single dose of CDDP (Morisaki et al., 2008; Solanki et al., 2014), none have reported the variability in the expression of the kidney transporters following multiple CDDP dose administrations. The results of this study suggest that the expression of uptake transporter in the kidney was unaffected by multiple CDDP dose administrations, while that of efflux transporters such as P-gp, Bcrp, and Mrp4 increased. As these transporters are generally involved in the recognition and efflux of anticancer agents (Yee et al., 2018), their expression might be increased by multiple administrations of CDDP. On the contrary, pharmacokinetics of CDDP may be unaffected because P-gp, Bcrp, and Mrp4 do not recognize CDDP as a substrate (Gupta et al., 2019; Ji et al., 2018). However, the pharmacokinetics of other anticancer drugs concomitantly administered with CDDP may be affected. In particular, anticancer drugs such as etoposide, methotrexate, and paclitaxel, which

are substrates for P-gp (Begicevic and Falasca, 2017), are frequently used in combination with CDDP, and therefore, it is necessary to evaluate the effect of changes in transporter expression due to multiple CDDP administrations, on the pharmacokinetics of other anticancer agents.

It has been reported that CDDP at a high dose affects the renal tissue and various markers, whereas CDDP at low doses, as in this study, alters only markers (Yokoo et al., 2007). We evaluated the histological images of the kidney tissue after hematoxylin-eosin staining and periodic acid-Schiff staining. There was no significant effect of CDDP on the renal tissue, because the dose of CDDP used in this study is low and equivalent to that in clinical practice (data not shown). In addition, we previously reported that the serum creatinine levels and Kim-1 are increased as well as Timp-1in a CIN model under the same conditions as this study (Saito et al., 2017a). Therefore, we consider that higher doses of CDDP and longer repeated administrations may cause obvious renal damage.

In the present study, we demonstrated that ascorbic acid suppressed the increase in P-gp expression and attenuated CIN. This may be due to reduction in oxidative stress in the kidney by ascorbic acid owing to its high superoxide scavenging potential. Although antioxidant substrates have been reported to reduce CIN (Sahin et al., 2014; Trujillo et al., 2016), evaluating the CIN-attenuating effect of ascorbic acid and its effect on P-gp expression via multiple low-dose administrations of CDDP has not been reported until now. In addition,

considering the anticancer activity of ascorbic acid (Shenoy et al., 2018), it can be expected that the antitumorigenic effect of CDDP will be enhanced while attenuating CIN, through co-administration of ascorbic acid. On the other hand, both P-gp expression and CIN are induced by ROS (Kawai et al., 2006; Ziemann et al., 1999), which in turn, is reduced by the antioxidant effect of ascorbic acid, but the fact that P-gp expression might decrease with CIN reduction cannot be ruled out.

CDDP has been reported to decrease Sod1 levels and increase Ho-1 expression in the kidney in a previous study (Holditch et al., 2019). Our results were consistent with those of this study, except that Sod1 expression was decreased only in the liver, and no effect was observed in the jejunum and ileum. CDDP-induced kidney injury is highly selective because it is a substrate for Oct2 which is specifically expressed in the kidney, hence renal uptake of CDDP takes place (Yonezawa et al., 2005). However, some amount of CDDP accumulates in the liver also via Oct1 (Bandu et al., 2015), which is mainly expressed in the liver and has a lower affinity for CDDP than Oct2 (Yonezawa et al., 2006). Oct1 might contribute to decreased Sod1 levels in the liver, and mild liver injury may be caused by CDDP administration. Moreover, CDDP may cause variability in the expression of hepatic transporters as well, however further evaluation is necessary.

In the present study, ascorbic acid was administered i.p., one hour before CDDP administration. In clinical practice, ascorbic acid is administered orally as well as in injection

form. However, due to its high water solubility (Tarng et al., 2001), administration by injection, as in this study, may be suitable to increase the blood concentration of ascorbic acid so that its maximal therapeutic effect on CIN can be achieved.

Ascorbic acid attenuated CIN and decreased P-gp expression in the kidney, but it did not affect CDDP-induced weight loss. This result is similar to that of magnesium as reported in our previous study (Saito et al., 2017a; Saito et al., 2017b). Although CDDP has some side effects such as nausea, vomiting, and anorexia (Oun et al., 2018), the effect of ascorbic acid is considered to be CIN-specific only.

5. Conclusion

We evaluated the variability in the expression of transporter in the kidney with multiple CDDP dose administrations, and demonstrated that the expression levels of P-gp, Bcrp, and Mrp4 were increased. Moreover, we revealed that ascorbic acid attenuated CIN and reduced P-gp expression, which was upregulated by CDDP. In conclusion, ascorbic acid can attenuate CIN and reverse P-gp-mediated changes in drug pharmacokinetics.

Declaration of interest

None declared.

Acknowledgements

This work was supported by the Japan Society for the Promotion of Science (JSPS; KAKENHI Grant Number 19K16437 and JP19J11051) and The Research Foundation for Pharmaceutical Sciences. We would like to thank Editage (www.editage.jp) for English language editing.

References

Abdel-Daim, M.M., Abushouk, A.I. Donia, T., Alarifi, S., Alkahtani, S., Aleya, L., Bumgau, S.G., 2019. The nephroprotective effects of allicin and ascorbic acid against cisplatin-induced toxicity in rats. Environ. Sci. Pollut. Res. Int. 26, 13502–13509. https://doi.org/10.1007/s11356-019-04780-4.

Bandu, R., Ahn, H.S., Lee, J.W., Kim, Y.W., Choi, S.H., Kim, H.J., Kim, K.P., 2015. Distribution study of cisplatin in rat kidney and liver cancer tissues by using liquid chromatography electrospray ionization tandem mass spectrometry. J. Mass Spectrom. 50, 844–853. https://doi.org/10.1002/jms.3594.

Begicevic, R., Falasca, M., 2017. ABC Transporters in Cancer Stem Cells: Beyond Chemoresistance. Int. J. Mol. Sci. 18, 2362. https://doi.org/10.3390/ijms18112362.

Casares, C., Ramírez-Camacho, R., Trinidad, A., Roldán, A., Jorge, E., García-Berrocal, J.R., 2012. Reactive oxygen species in apoptosis induced by cisplatin: review of physiopathological mechanisms in animal models. Eur. Arch. Otorhinolaryngol. 269, 2455–2459. https://doi.org/10.1007/s00405-012-2029-0.

de Jongh, F.E., van Veen, R.N., Veltman, S.J., de Wit, R., van der Burg, M.E., van den Bent, M.J., Planting, A.S., Graveland, W.J., Stoter, G., Verweij, J., 2003. Weekly high-dose cisplatin is a feasible treatment option: analysis on prognostic factors for toxicity in 400 patients. Br. J. Cancer 88, 1199–1206. https://doi.org/10.1038/sj.bjc.6600884.

Gupta, P., Gao, H., Ashar, Y.V., Karadkhelkar, N.M., Yoganathan, S., Chen, Z., 2019. Ciprofloxacin Enhances the Chemosensitivity of Cancer Cells to ABCB1 Substrates. Int. J. Mol. Sci. 20, 268. https://doi.org/10.3390/ijms20020268.

Holditch, S.J., Brown, C.N., Lombardi, A.M., Nguyen, K.N., Edelstein, C.L., 2019. Recent Advances in Models, Mechanisms, Biomarkers, and Interventions in Cisplatin-Induced Acute Kidney Injury. Int. J. Mol. Sci. 20, 3011. https://doi.org/10.3390/ijms20123011.

Ji, N., Yang, Y., Cai, C., Lei, Z., Wang, J., Gupta, P., Teng, Q., Chen, Z., Kong, D., Yang, D.,

2018. VS-4718 Antagonizes Multidrug Resistance in ABCB1- and ABCG2-Overexpressing Cancer Cells by Inhibiting the Efflux Function of ABC Transporters. Front. Pharmacol. 9, 1236. https://doi.org/10.3389/fphar.2018.01236.

Kawai, Y., Nakao, T., Kunimura, N., Kohda, Y., Gemba, M., 2006. Relationship of intracellular calcium and oxygen radicals to Cisplatin-related renal cell injury. J. Pharmacol. Sci. 100, 65–72. https://doi.org/10.1254/jphs.fp0050661.

Miller, R.P., Tadagavadi, R.K., Ramesh, G., Reeves, W.B., 2010. Mechanisms of Cisplatin nephrotoxicity. Toxins (Basel) 2, 2490–2518. https://doi.org/10.3390/toxins2112490.

Morisaki, T., Matsuzaki, T., Yokoo, K., Kusumoto, M., Iwata, K., Hamada, A., Saito, H., 2008. Regulation of renal organic ion transporters in cisplatin-induced acute kidney injury and uremia in rats. Pharm. Res. 25, 2526–2533. https://doi.org/10.1007/s11095-008-9668-2.

Okamoto, K., Saito, Y., Narumi, K., Furugen, A., Iseki, K., Kobayashi, M., 2020. Comparison of the nephroprotective effects of non-steroidal anti-inflammatory drugs on cisplatin-induced nephrotoxicity in vitro and in vivo. Eur. J. Pharmacol. 884, 173339. https://doi.org/10.1016/j.ejphar.2020.173339.

Oun, R., Moussa, Y.E., Wheate, N.J., 2018. The side effects of platinum-based chemotherapy drugs: a review for chemists. Dalton Trans. 47, 6645–6653. https://doi.org/10.1039/c8dt00838h.

Pabla, N., Dong, Z., 2008. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int. 73, 994–1007. https://doi.org/10.1038/sj.ki.5002786.

Padayatty, S.J., Levine, M., 2016. Vitamin C: the known and the unknown and Goldilocks. Oral Dis. 22, 463–493. https://doi.org/10.1111/odi.12446.

Park, M.S., De Leon, M., Devarajan, P., 2002. Cisplatin induces apoptosis in LLC-PK1 cells via activation of mitochondrial pathways. J. Am. Soc. Nephrol. 13, 858–865.

Prestayko, A.W., D'Aoust, J.C., Issell, B.F., Crooke, S.T., 1979. Cisplatin (cis-diamminedichloroplatinum II). Cancer Treat. Rev. 6, 17–39. https://doi.org/10.1016/s0305-7372(79)80057-2.

Rosenberg, B., VanCamp, L., Trosko, J.E., Mansour, V.H., 1969. Platinum compounds: a new class of potent antitumour agents. Nature 222, 385–386. https://doi.org/10.1038/222385a0.

Sahin, K., Orhan, C., Tuzcu, M., Muqbil, I., Sahin, N., Gencoglu, H., Guler, O., Padhye, S.B., Sarkar, F.H., Mohammad, R.M., 2014. Comparative in vivo evaluations of curcumin and its analog difluorinated curcumin against cisplatin-induced nephrotoxicity. Biol. Trace Elem. Res. 157, 156–163. https://doi.org/10.1007/s12011-014-9886-x.

Saito, Y., Okamoto, K., Kobayashi, M., Narumi, K., Furugen, A., Yamada, T., Iseki, K., 2017a. Magnesium co-administration decreases cisplatin-induced nephrotoxicity in the multiple cisplatin administration. Life Sci. 189, 18–22.

https://doi.org/10.1016/j.lfs.2017.08.028.

Saito, Y., Okamoto, K., Kobayashi, M., Narumi, K., Yamada, T., Iseki, K., 2017b. Magnesium attenuates cisplatin-induced nephrotoxicity by regulating the expression of renal transporters. Eur. J. Pharmacol. 811, 191–198. https://doi.org/10.1016/j.ejphar.2017.05.034.

Shenoy, N., Creagan, E., Witzig, T., Levine, M., 2018. Ascorbic Acid in Cancer Treatment: Let the Phoenix Fly. Cancer Cell 34, 700–706. https://doi.org/10.1016/j.ccell.2018.07.014.

Solanki, M.H., Chatterjee, P.K., Gupta, M., Xue, X., Plagov, A., Metz, M.H., Mintz, R., Singhal, P.C., Metz, C.N., 2014. Magnesium protects against cisplatin-induced acute kidney injury by regulating platinum accumulation. Am. J. Physiol. Renal. Physiol. 307, F369–F384. https://doi.org/10.1152/ajprenal.00127.2014.

Tarng, D.C., Huang, T.P., Wei, Y.H., 2001. Erythropoietin and iron: the role of ascorbic acid. Nephrol. Dial. Transplant. 16, 35–39. https://doi.org/10.1093/ndt/16.suppl_5.35.

Trujillo, J., Molina-Jijón, E., Medina-Campos, O.N., Rodríguez-Muñoz, R., Reyes, J.L., Loredo, M.L., Barrera-Oviedo, D., Pinzón, E., Rodríguez-Rangel, D.S., Pedraza-Chaverri, J., 2016. Curcumin prevents cisplatin-induced decrease in the tight and adherens junctions: relation to oxidative stress. Food Funct. 7, 279–293. https://doi.org/10.1039/c5fo00624d.

Yee, S.W., Brackman, D.J., Ennis, E.A., Sugiyama, Y., Kamdem, L.K., Blanchard, R., Galetin, A., Zhang, L., Giacomini, K.M., 2018. Influence of Transporter Polymorphisms on Drug Disposition and Response: A Perspective from the International Transporter Consortium. Clin. Pharmacol. Ther. 104, 803–817. https://doi.org/10.1002/cpt.1098.

Yokoo, S., Yonezawa, A., Masuda, S., Fukatsu, A., Katsura, T., Inui, K., 2007. Differential contribution of organic cation transporters, OCT2 and MATE1, in platinum agent-induced nephrotoxicity. Biochem. Pharmacol. 74, 477–487. https://doi.org/10.1016/j.bcp.2007.03.004.

Yonezawa, A., Masuda, S., Nishihara, K., Yano, I., Katsura, T., Inui, K., 2005. Association between tubular toxicity of cisplatin and expression of organic cation transporter rOCT2 (Slc22a2) in the rat. Biochem. Pharmacol. 70, 1823–1831. https://doi.org/10.1016/j.bcp.2005.09.020.

Yonezawa, A., Masuda, S., Yokoo, S., Katsura, T., Inui, K., 2006. Cisplatin and oxaliplatin, but not carboplatin and nedaplatin, are substrates for human organic cation transporters (SLC22A1-3 and multidrug and toxin extrusion family). J. Pharmacol. Exp. Ther. 319, 879–886. https://doi.org/10.1124/jpet.106.110346.

Ziemann, C., Bürkle, A., Kahl, G.F., Hirsch-Ernst, K.I., 1999. Reactive oxygen species participate in mdr1b mRNA and P-glycoprotein overexpression in primary rat hepatocyte cultures. Carcinogenesis 20, 407–414. https://doi.org/10.1093/carcin/20.3.407.

Figure Legends

Fig. 1: Effect of multiple-dose CDDP administration on kidney injury and antioxidant markers. The expression levels of (A) Timp-1, (B) Sod1, and (C) Ho-1 mRNA were normalized to Actin. The expression level of the control group was arbitrarily set at 1.0. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group; unpaired Student's *t*-test. Data are presented as mean with standard deviation (S.D.), n = 5–6 per group.

Fig. 2: Effect of multiple CDDP dose administrations on renal transporter expression. (A) Uptake transporters and Actin, (C) efflux transporters and Actin protein expression by western blotting, and (B, D) expression levels were quantified by ImageJ analysis software, respectively. Each transporter was normalized to Actin. The expression level of the control group was arbitrarily set at 1.0. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group; unpaired Student's t-test. Data are presented as mean with S.D., n = 6 per group.

Fig. 3: Effect of ascorbic acid on CDDP–induced cytotoxicity in NRK-52E cells. (A) NRK-52E cells were treated with CDDP (0–60 μM) with or without ascorbic acid (500 μM) for 48 h. Data are presented as mean with standard error of the mean (S.E.M.) from three independent experiments. (B) Ascorbic acid and N-acetylcysteine were dissolved in water. Chemiluminescence of the control group was arbitrarily set at 100%. $^{**}P < 0.01$ compared with the control group, $^{††}P < 0.01$ compared with the N-acetylcysteine group; Tukey's post-hoc test. Data are presented as mean with S.D., n = 5 per group.

Fig. 4: Effect of co-administration of ascorbic acid and CDDP on rats. (A) The body weight of each day was calculated by subtracting from the body weight of day 1. (B) The serum creatinine level was measured on day 22. (C) Sod1 mRNA expression level was normalized to Actin. The expression level of the control group was arbitrarily set at 1.0. (D) P-gp and Actin protein expression by western blotting, and (E) expression levels were quantified by ImageJ analysis software. P-gp was normalized to Actin. The expression level of the control group was arbitrarily set at 1.0. $^{**}P < 0.01$ compared with the control group, $^{††}P < 0.01$ compared with the ascorbic acid group, $^{‡}P < 0.05$ and $^{‡‡}P < 0.01$ compared with the CDDP group; Tukey's post-hoc test. Data are presented as mean with S.D., n = 5–6 per group.

Table 1. Primer Sequences

Genes	Forward Sequence	Reverse Sequence
Timp-1	5-ttgcttgtggacagatcaga-3	5-gtattgccaggtgcacaaat-3
Sod1	5-tgcaggacctcattttaatcct-3	5-tccagcatttccagtctttgta-3
Ho-1	5-ctttcagaagggtcaggtgtc-3	5-tgcttgtttcgctctatctcc-3
Actin	5-ctatcggcaatgagcggttc-3	5-gaggtctttacggatgtcaacg-3

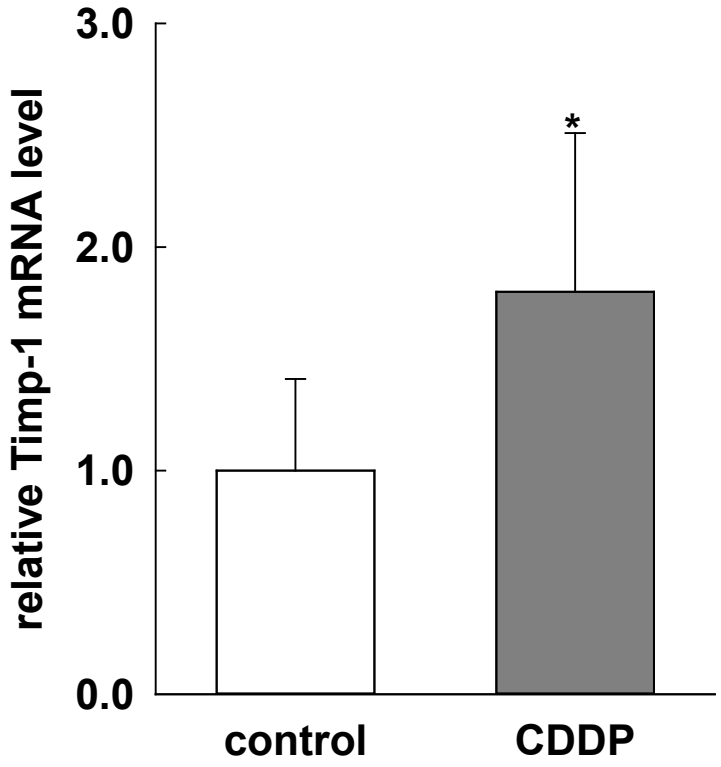

Fig. 1A

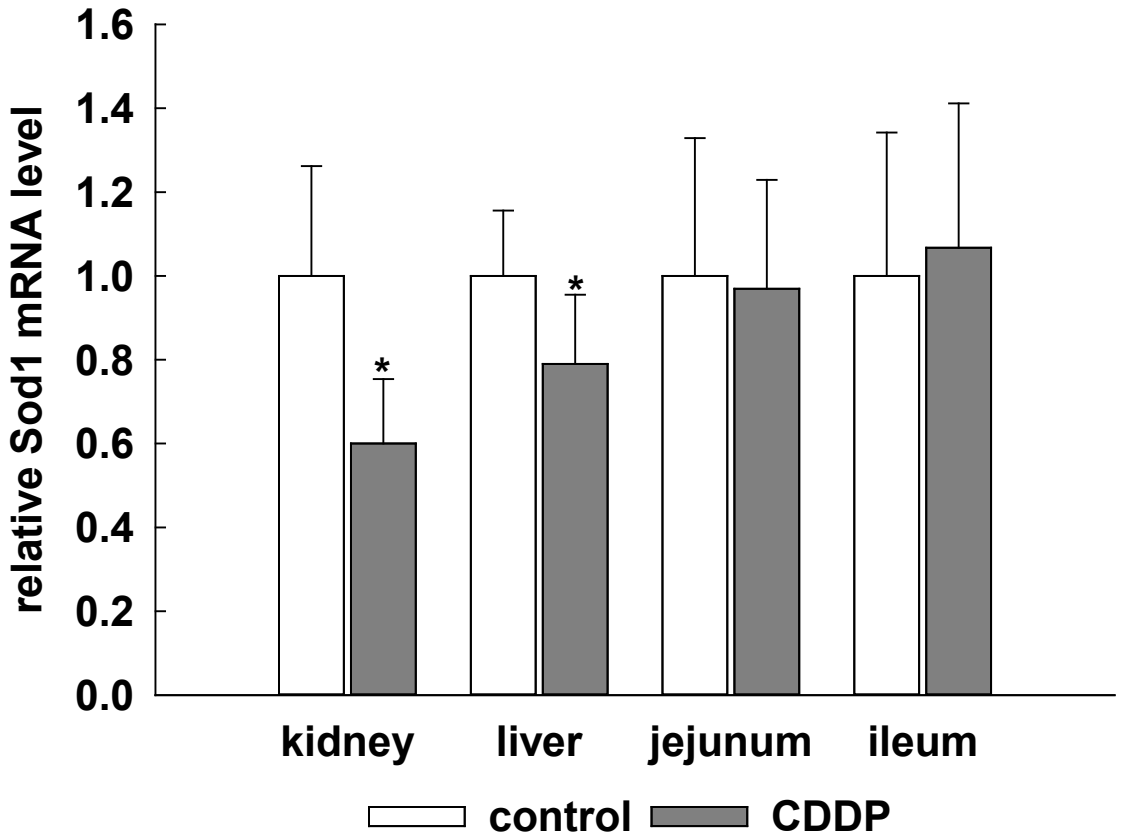
relative Sod1 mRNA level
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
kidney
liver
jejunum
ileum
control
CDDP

Fig. 1B

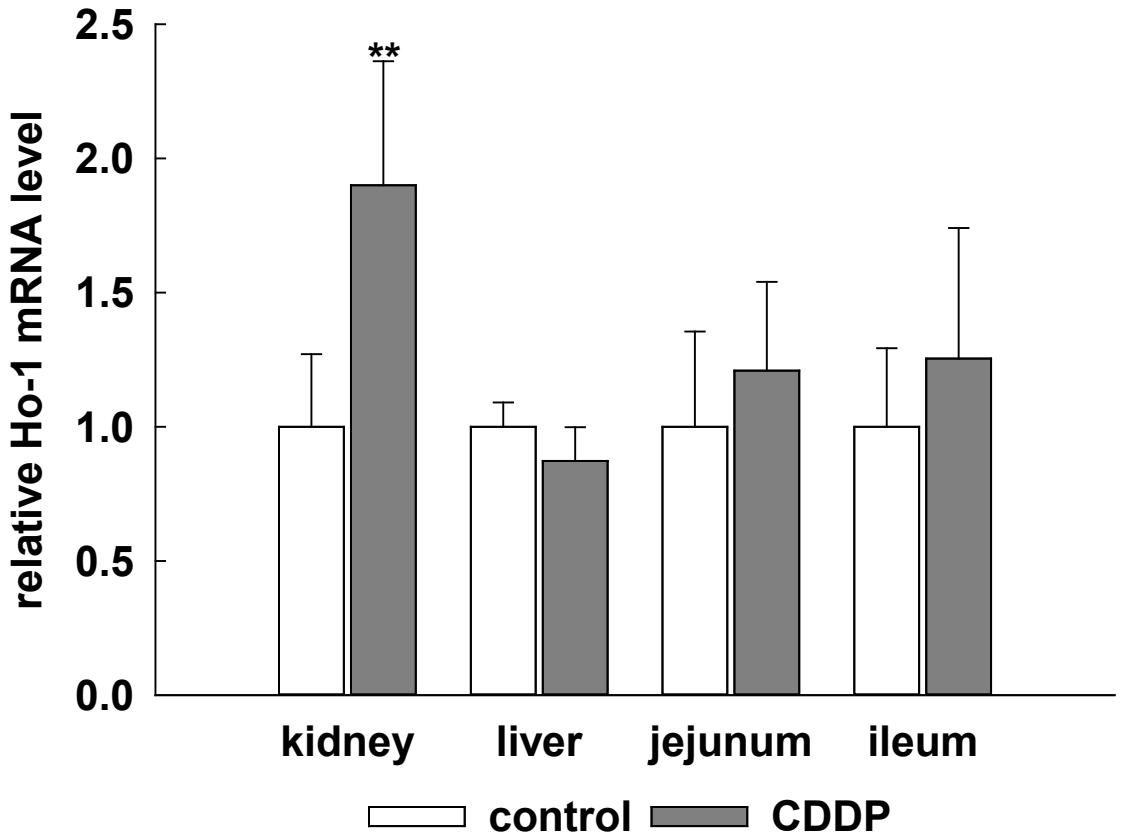
relative Ho-1 mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
**
kidney
liver
jejunum
ileum
control
CDDP

Fig. 1C

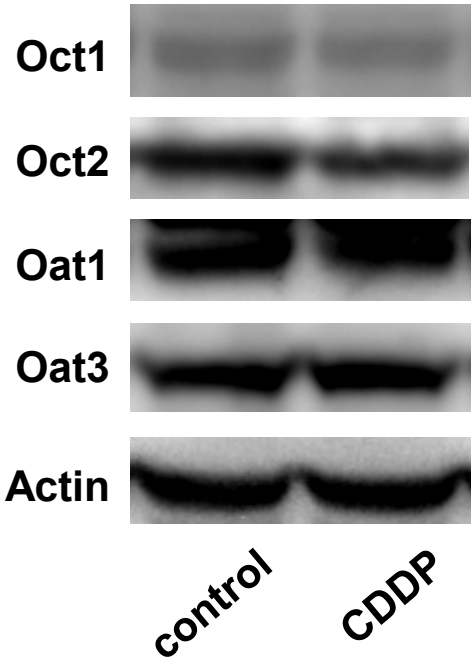

Fig. 2A

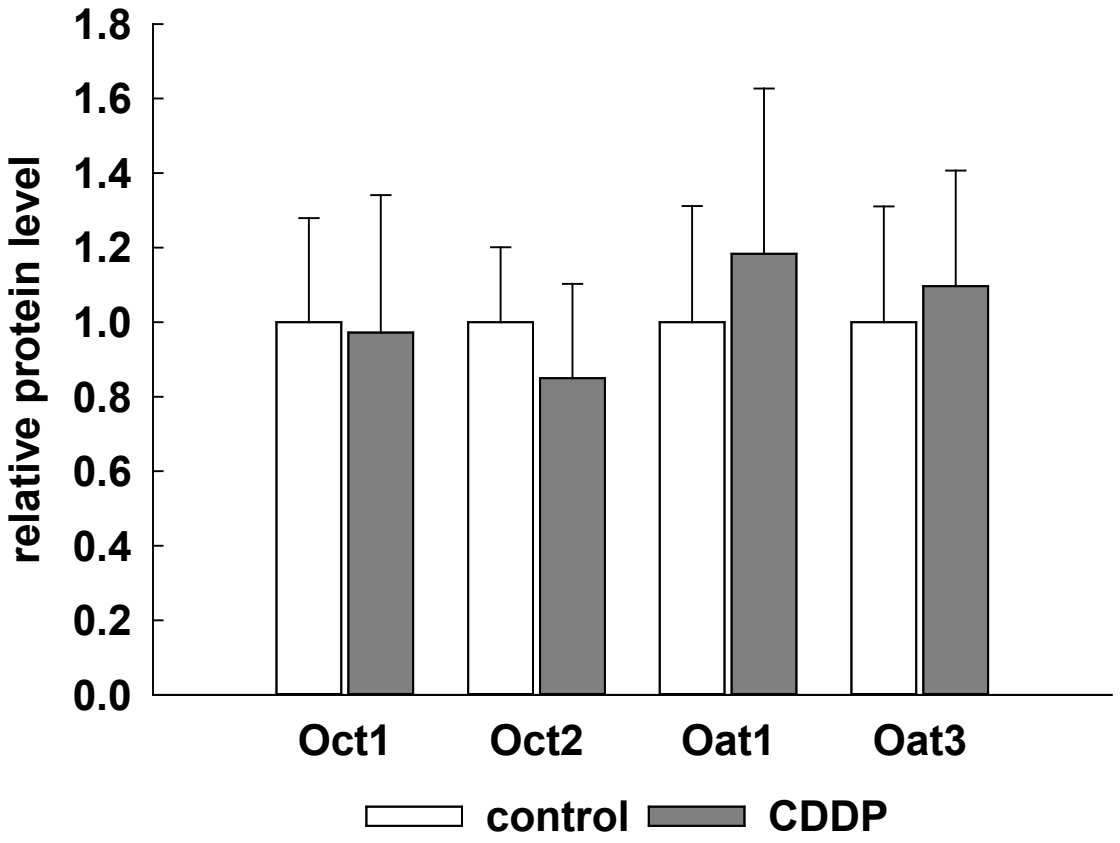
relative protein level
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Oct1
Oct2
Oat1
Oat3
control
CDDP

Fig. 2B

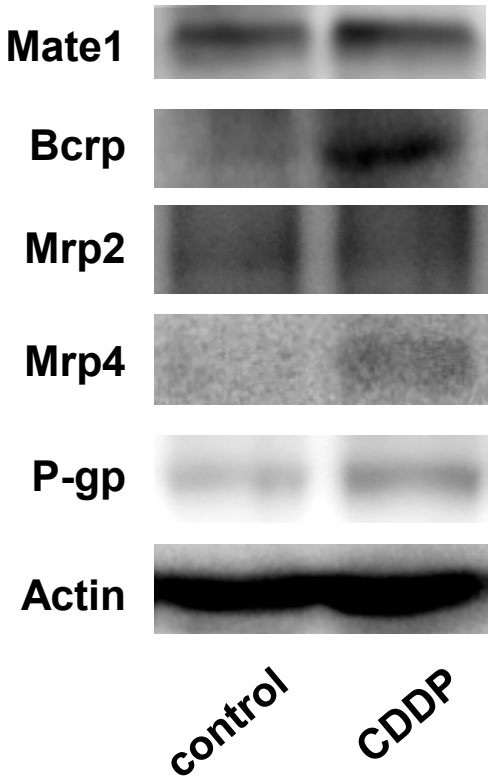

Fig. 2C

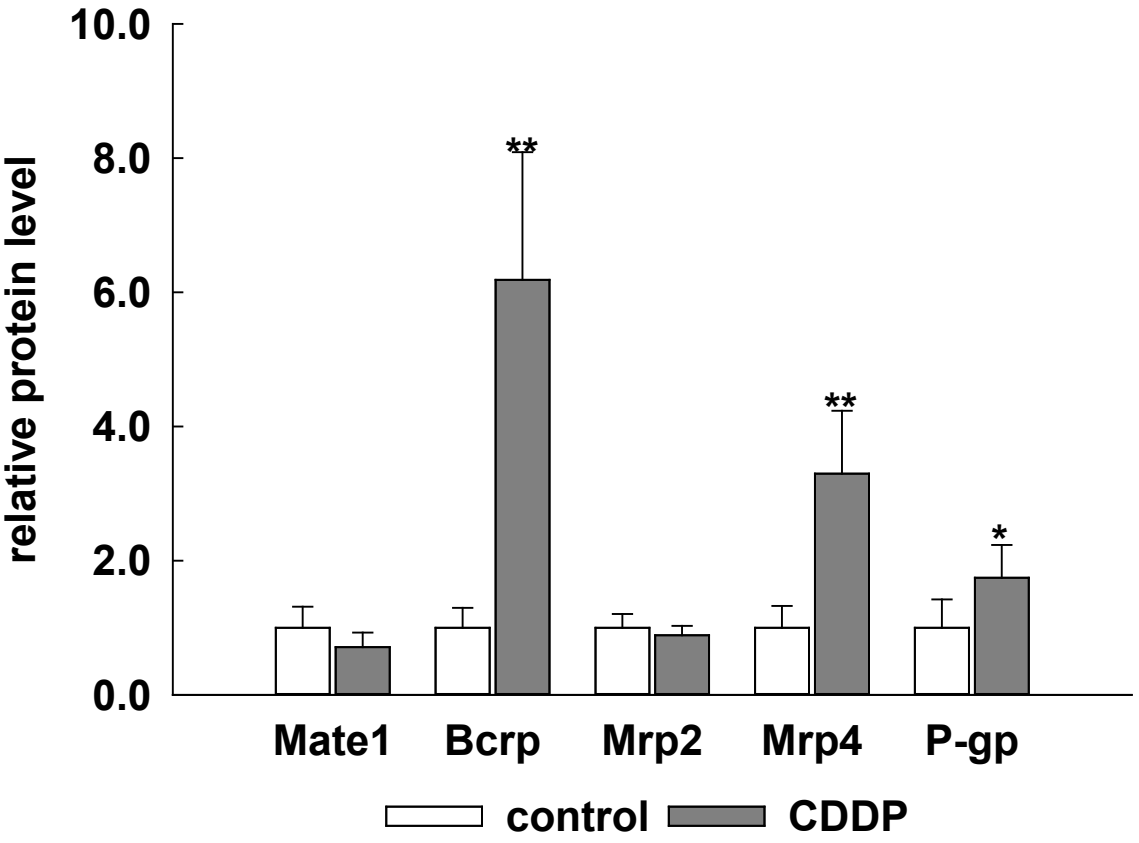
10.0
8.0
6.0
4.0
2.0
0.0
relative protein level
**
**
*
Mate1
Bcrp
Mrp2
Mrp4
P-gp
control
CDDP

Fig. 2D

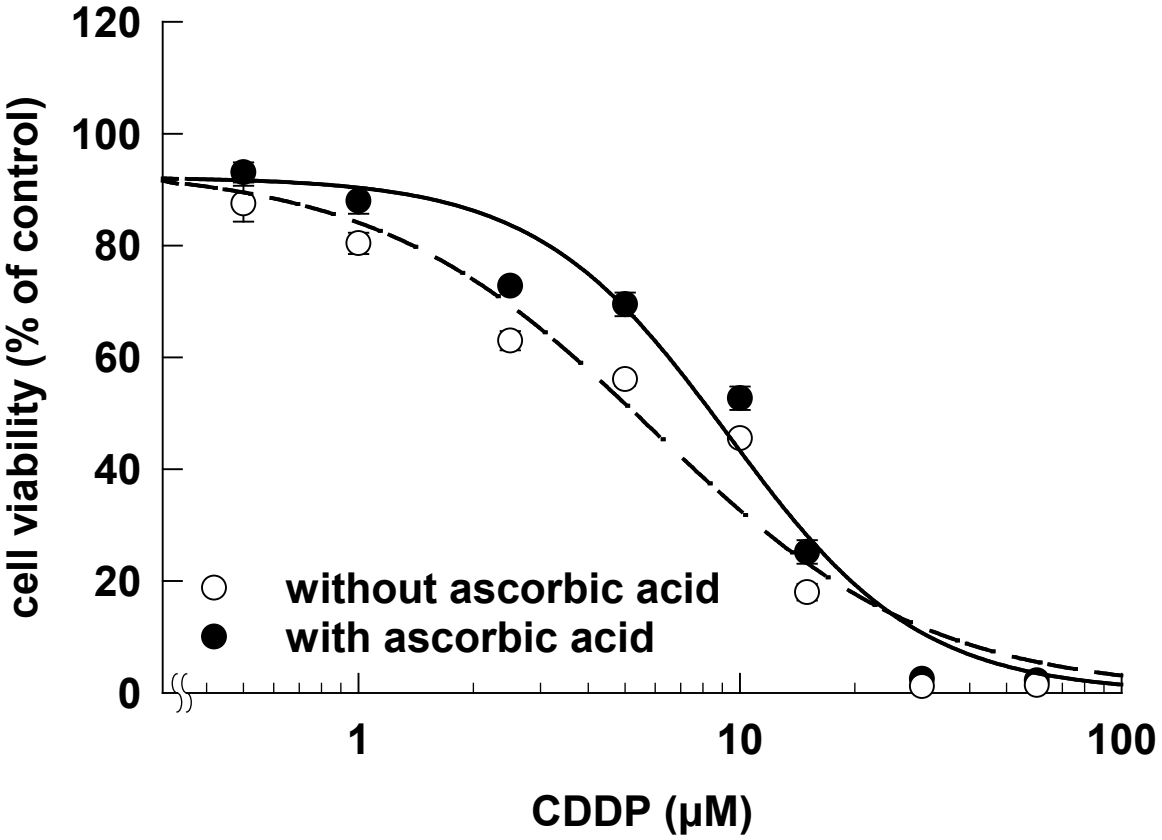
120
100
80
60
40
20
0
cell viability (% of control)
without ascorbic acid
with ascorbic acid
1
10
100
CDDP (μM)

Fig. 3A

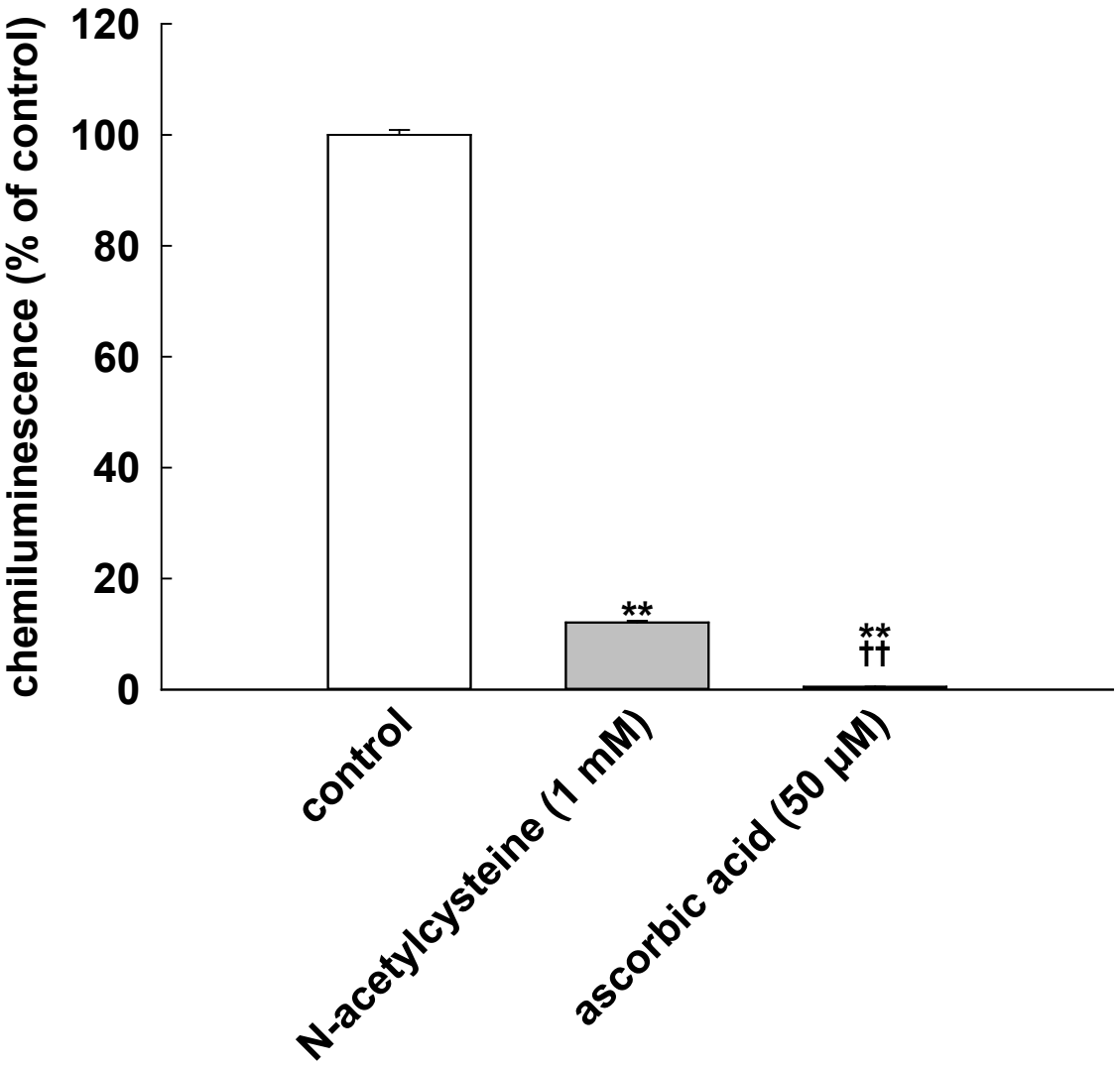
chemiluminescence (% of control)
120
100
80
60
40
20
0
**
**
††
control
N-acetylcysteine (1 mM)
ascorbic acid (50 μM)

Fig. 3B

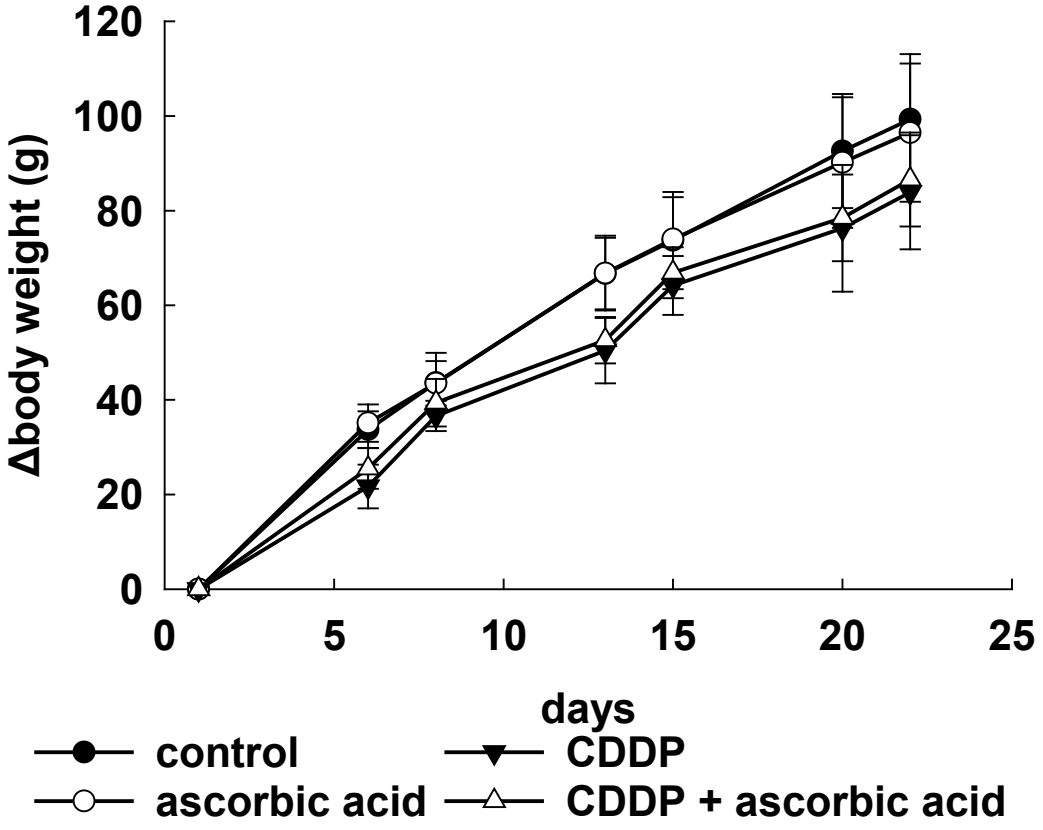
120
100
80
60
40
20
0
Δbody weight (g)
0
5
10
15
20
25
days
control
CDDP
ascorbic acid
CDDP + ascorbic acid

Fig. 4A

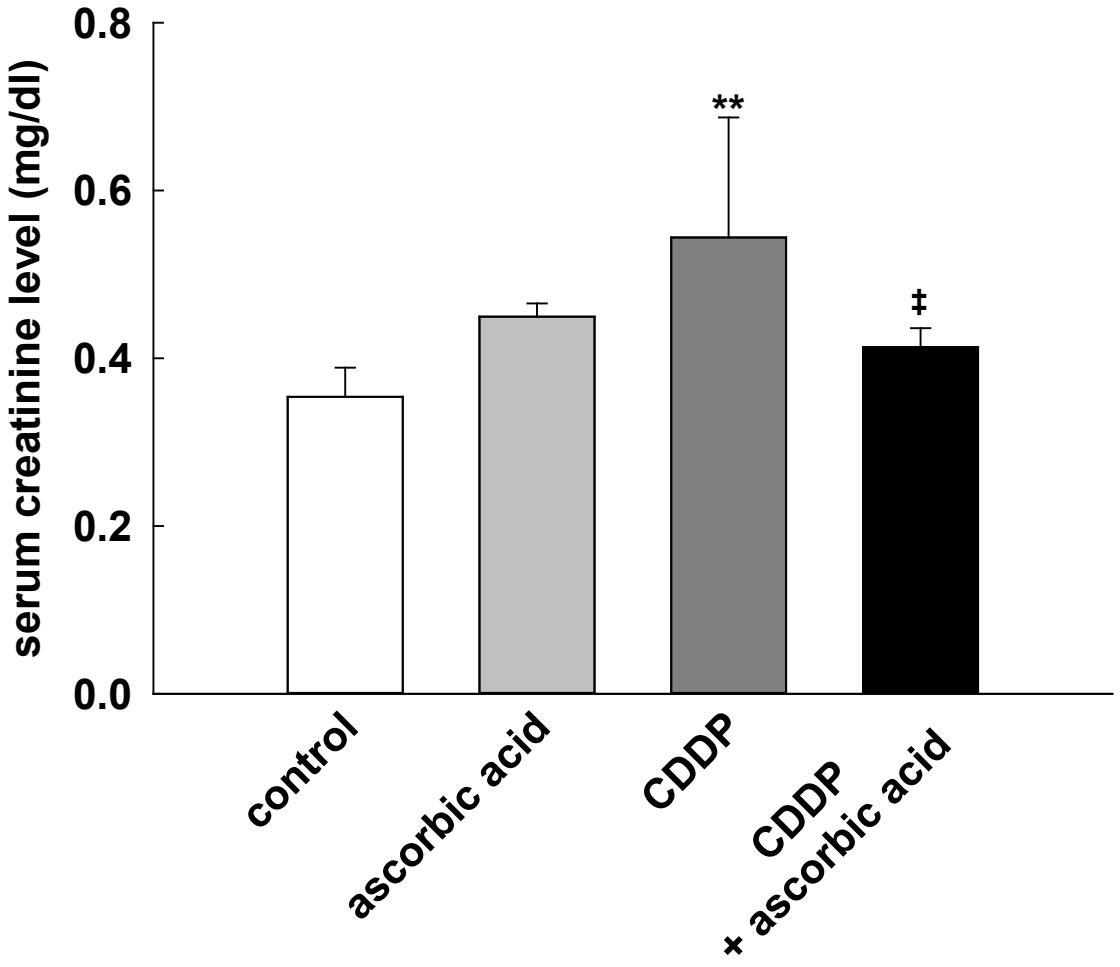
0.8
0.6
0.4
0.2
0.0
serum creatinine level (mg/dl)
**
‡
control
ascorbic acid
CDDP
CDDP
+ ascorbic acid

Fig. 4B

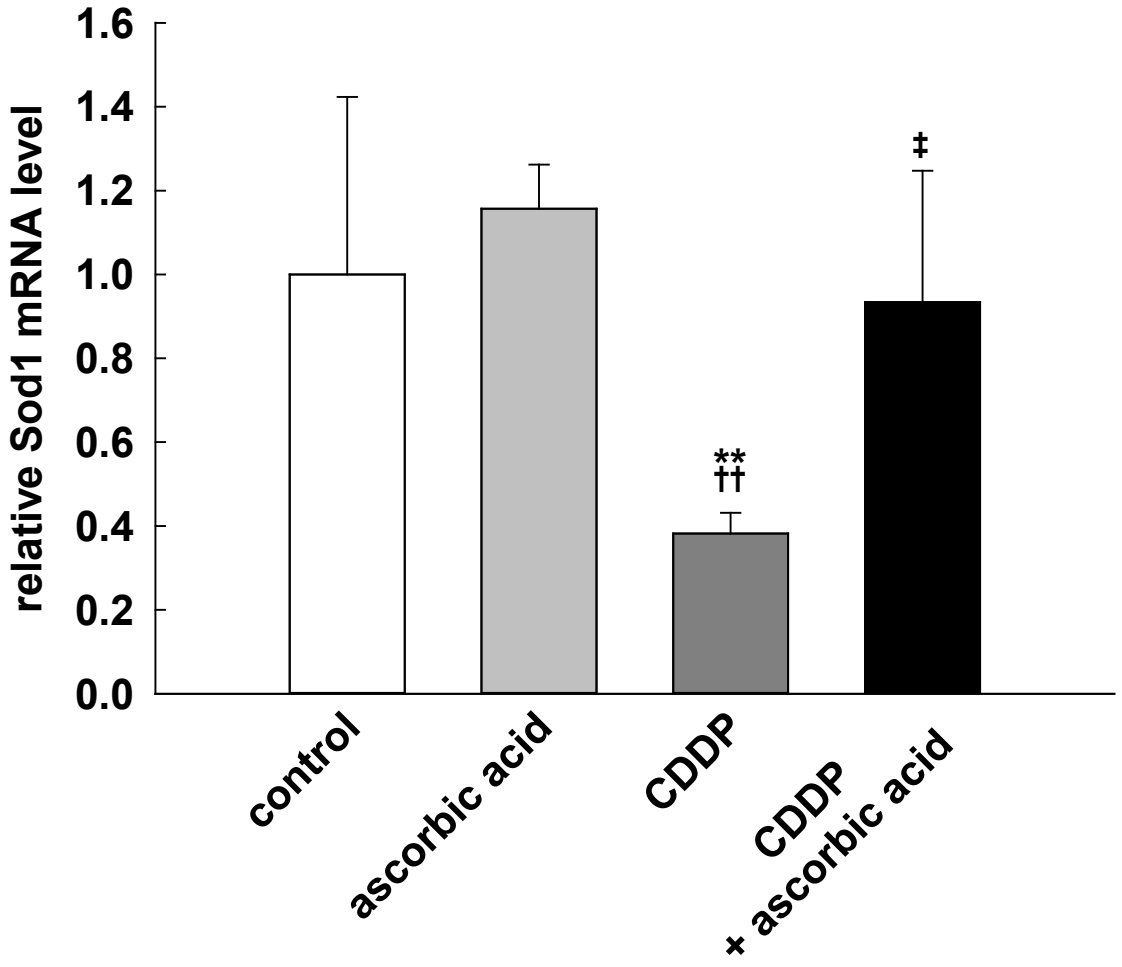
relative Sod1 mRNA level
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
††
‡
control
ascorbic acid
CDDP
CDDP
+ ascorbic acid

Fig. 4C

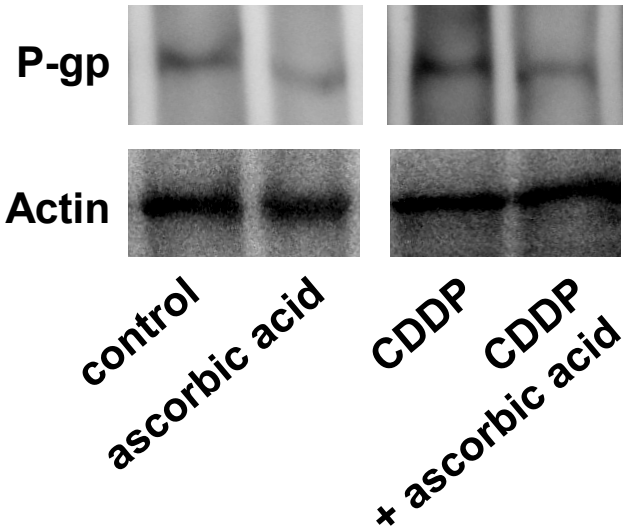

Fig. 4D

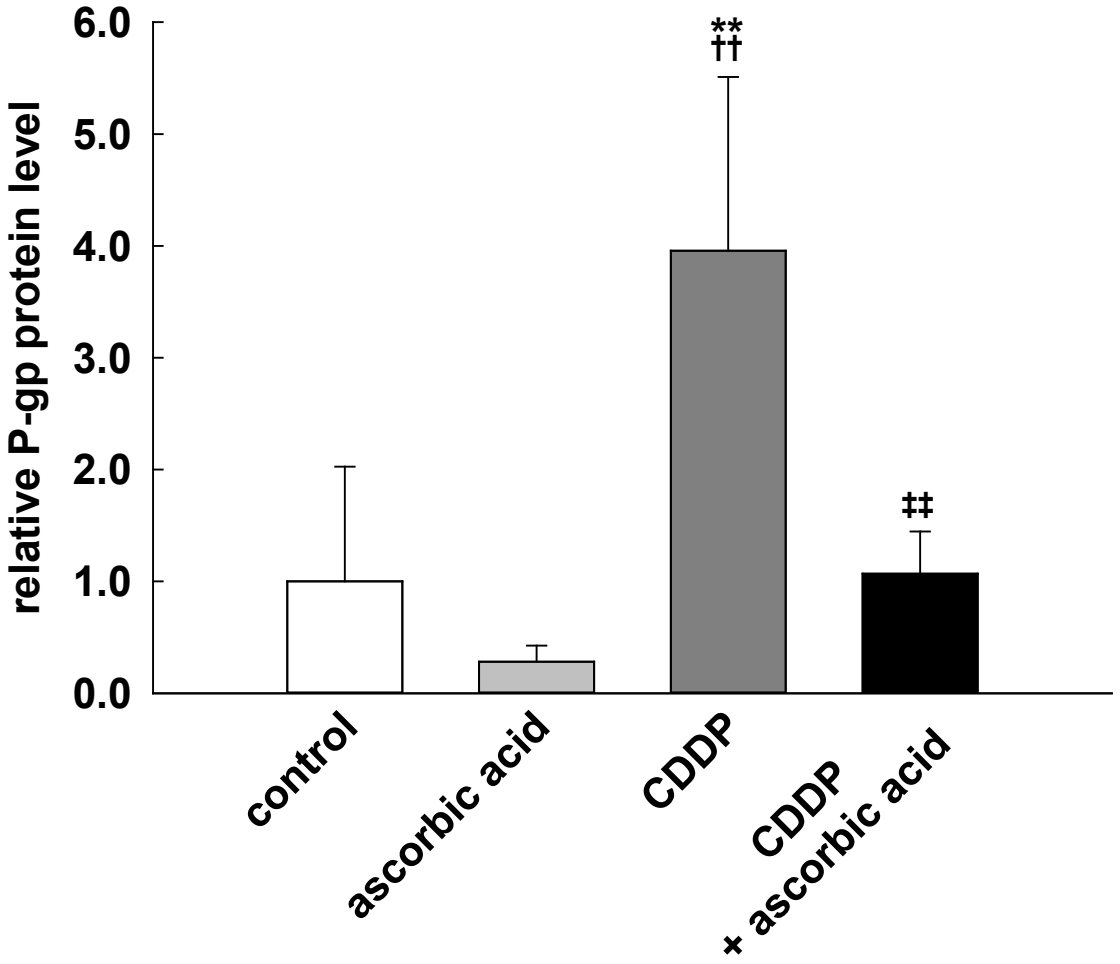
relative P-gp protein level
6.0
5.0
4.0
3.0
2.0
1.0
0.0
**
††
‡‡
control
ascorbic acid
CDDP
CDDP
+ ascorbic acid

Fig. 4E