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Original Research

Renin-angiotensin system inhibitors combined with cisplatin exacerbate cisplatin-induced nephrotoxicity in mice

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ABSTRACT

Introduction: We previously reported that the concomitant use of enalapril and telmisartan exacerbates the risk of cisplatin (CDDP)-induced acute renal dysfunction compared to other antihypertensive drugs in mice. Thus, in the current study, we investigated the risk of developing chronic kidney disease following repeated concomitant use of CDDP and antihypertensive drugs.

Materials and Methods: Male BALB/c mice were divided into 12 groups: (1) Control group (untreated), (2) CDDP group (7 mg/kg, CDDP), (3) AML group (5 mg/kg, amlodipine), (4) ENA group (2.5 mg/kg, enalapril), (5) TEL group (10 mg/kg, telmisartan), (6) LOS group (10 mg/kg, losartan), (7) CDDP+AML group (5 mg/mL, AML), (8) CDDP+ENA group (2.5 mg/kg, ENA), (9) CDDP+LowENA group (1.25 mg/kg, ENA), (10) CDDP+TEL group (10 mg/kg, TEL), (11) CDDP+LowTEL group (5 mg/kg, TEL), and (12) CDDP+LOS group (10 mg/kg, LOS). CDDP was administered intraperitoneally four times every 7 days, and each antihypertensive drug was administered orally from day 3 before CDDP administration until day 24 (six times a week). The degree of renal damage was assessed. The nephrotoxicity of each individual was evaluated by measuring serum creatinine and blood urea nitrogen levels. The degrees of renal fibrosis and epithelial-mesenchymal transition were also examined in kidney tissue sections.

Results and Discussion: The results suggest that combinatorial treatment of CDDP and renin-angiotensin system inhibitors, particularly ENA and TEL, may exacerbate CDDP-induced nephrotoxicity. This study clearly demonstrates the need for large-scale clinical studies to construct treatment regimens that do not interfere with the therapeutic intensity of CDDP.

Introduction

Cisplatin (cis-diamminedichloroplatinum, CDDP) is a platinum-based drug widely used in chemotherapy for various cancers that binds to the purine bases (guanine and adenine) in cancer cell DNA, thereby forming cross-links to inhibit DNA replication and transcription and producing an anti-tumor effect. However, it has been reported that 245 (31.5%) of 777 patients treated with CDDP developed acute kidney injury (AKI) [1]. Meanwhile, AKI can be avoided with massive fluid replacement and forced diuresis before and after CDDP administration [2]. Thus, CDDP was approved in Canada, the United States, and Italy in 1978 and Japan in 1983.

As general prophylaxis for renal injury, the administration of 2.5 L or more of fluid and, if necessary, the use of diuretics is recommended. However, 41% of 400 patients treated with a high dose of CDDP (70–85 mg/m^2) reportedly had elevated serum creatinine levels even with fluid replacement [3]. Horinouchi et al. [4] reported that although short hydration was effective in patients with lung cancer, it was unclear whether all patients benefit from this regimen as the requirements for short hydration are a performance status of 0 or 1 and creatinine clearance of at least 60 mL/min. Moreover, CDDP-induced AKI can lead to irreversible chronic tubulopathy and renal fibrosis, which can progress to chronic kidney disease (CKD) [5,6]. Indeed, nephrotoxicity is a dose-limiting factor for CDDP, which induces renal injury by promoting cytochrome c release from proximal tubular epithelial cells and activating caspase-9 [7]. Additionally, endothelial dysfunction and vasoconstriction cause direct tubular epithelial cell toxicity, resulting in decreased renal blood flow [8,9].

Furthermore, antihypertensive drugs or low blood pressure increase the risk of developing AKI. In fact, Arora et al. [10] reported that

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renin-angiotensin system (RAS) inhibitors increased the risk of developing AKI by 27.6% in patients after cardiac surgery. Moreover, the risk of developing AKI is approximately doubled in septic patients using RAS inhibitors [11,12]. In contrast, Molinas et al. [13] reported that the pre-administration of losartan (LOS) had a kidney-protective effect on ischemic AKI, and Brar et al. [14] reported that the use of RAS inhibitors in patients with AKI reduced total mortality. Furthermore, distinct RAS inhibitors may affect AKI differently. Therefore, there is a strong clinical need to elucidate the pathogenesis of renal injury and to develop preventative dosing regimens. CDDP-induced AKI is exacerbated by the concomitant use of antihypertensive drugs, particularly RAS inhibitors and CDDP. However, clinically, chemotherapy regimens require a period of rest and repeated administration. Therefore, it is necessary to investigate the effect of antihypertensive drugs on CDDP-induced nephrotoxicity under repeated administration.

We are currently researching the construction of optimal treatment methods, including the avoidance of adverse effects in cancer chemotherapy [15,16]. Accordingly, the current study investigates the effects of antihypertensive drugs on CDDP-induced chronic kidney injury via repeated administration of CDDP to mice to mimic clinical practices.

Methods

Experimental animals

BALB/c mice (6 weeks old, male) were purchased from Japan SLC, Inc. (Shizuoka, Japan), housed in a conventional environment (room temperature 23 ± 1 °C, relative humidity 47–67%, light/dark cycle: 12 h), and provided food (CRF-1, gamma-irradiated feed, Oriental Bio Co., Ltd., Kyoto, Japan) and water *ad libitum*. This study was conducted in accordance with the Guidelines for the Appropriate Conduct of Animal Experiments (Science Council of Japan: June 1, 2006) and the regulations on animal experiments of Setsunan University (Permission number: K19–16).

Drugs

CDDP (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved to a concentration of 0.5 mg/mL using physiological saline solution (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan). Amlodipine besylate (AML; Norvasc®, Pfizer Japan, Inc., Tokyo, Japan), enalapril maleate (ENA; Renivace®, MSD K.K., Tokyo, Japan), telmisartan (TEL; Micardis®, Nippon Boehringer Ingelheim Co., Ltd., Tokyo, Japan), and LOS (Nu-lotan®, MSD K.K.) were dissolved, or suspended, in water for injection (Otsuka Pharmaceutical Factory Inc.) at concentrations of 5 mg/mL, 2.5 mg/mL, 10 mg/mL, and 10 mg/mL, respectively.

Experimental protocols

BALB/c mice were divided into the following 12 groups: (1) Control group ($n = 5$), (2) CDDP group (7 mg/kg, CDDP, $n = 5$), (3) AML group (5 mg/kg, $n = 5$), (4) ENA group (2.5 mg/kg, $n = 5$), (5) TEL group (10 mg/kg, $n = 5$), (6) LOS group (10 mg/kg, $n = 5$), (7) CDDP+AML group ($n = 5$), (8) CDDP+ENA group ($n = 10$), (9) CDDP+LowENA group (1.25 mg/kg, $n = 5$), (10) CDDP+TEL group ($n = 8$), (11) CDDP+LowTEL group (5 mg/kg, $n = 5$), and (12) CDDP+LOS group (10 mg/kg, $n = 5$). The degree of renal injury was examined. CDDP was administered intraperitoneally four times on days 0, 7, 14, and 21; each antihypertensive drug was administered orally beginning 3 days before CDDP administration (day −3) until day 24 (six times per week). Systolic blood pressure (sBP), serum creatinine (CRE), blood urea nitrogen (BUN), and serum albumin (ALB) levels were measured at days −3, 0, 7, 14, 21, and 25. At the end of the observation period (day 25), mice were lethally anesthetized and their kidneys were harvested.

Measurement of sBP

sBP was measured using a blood pressure monitor for mice and rats (MK-2000, Muromachi Kikai Co., Ltd., Tokyo, Japan).

Blood collection and serum separation

Blood was collected from the tail vein using a hematocrit capillary tube. Samples were then kept at 4 °C for 1 h and centrifuged at 12,000 rpm for 5 min using a hematocrit centrifuge to obtain serum and stored at −20 °C.

Measurement of CRE, BUN, and ALB concentrations

CRE, BUN, and ALB serum concentrations were measured using a SPOTCHEM system (ARKRAY, Inc., Kyoto, Japan). The reagent card used was SpotChem™II Renal Function-2 (ARKRAY, Inc.).

Kidney harvesting

Mice were anesthetized, and after confirming the absence of righting reflex, the abdomen was incised. After perfusion with phosphate-buffered saline (PBS) using a pump, the kidneys were harvested and placed in 4% paraformaldehyde/phosphate buffer (pH 7.0, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) at 4 °C overnight.

Assessment of renal fibrosis area (Masson trichrome staining)

The collected kidneys were embedded in paraffin according to conventional methods. The embedded tissues were cut into 3 μm sections using a microtome (ROM-380, Yamato Kohki Industrial Co., Ltd., Tokyo, Japan) and deparaffinized using xylene. Deparaffinized tissue sections were rinsed with running water, reacted with the first mordant solution (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for 20 min, and rinsed with running water for 3 min. The sections were then incubated with Meyer hematoxylin solution (FUJIFILM Wako Pure Chemical Corporation) for 5 min and washed under running water for 5 min. A second mordant solution (Muto Pure Chemicals Co., Ltd.) was added dropwise and reacted for 30 s, followed by rinsing with running water for 1 min. Tissue sections were then immersed in 1% acetic acid solution five times, reacted with 0.75% orange G solution (Muto Pure Chemicals Co., Ltd.) for 1 min, and immersed in 1% acetic acid solution five times. Next, tissues were immersed in Masson's dye solution B (Muto Pure Chemicals Co., Ltd.) for 5 min and subsequently immersed in 1% acetic acid solution five times. The specimens were then immersed in 2.5% phosphotungstic acid solution (Muto Pure Chemicals Co., Ltd.) for 15 min and immersed in 1% acetic acid solution five times. After reacting with aniline blue solution (Muto Pure Chemicals Co., Ltd.) for 30 min, the samples were immersed in 1% acetic acid solution five times. The kidney sections were dehydrated in ethanol, mounted with Canada balsam (FUJIFILM Wako Pure Chemical Corporation), and examined microscopically (OLYMPUS BX50, Tokyo, Japan). The glomerular area was calculated from the stained images and the renal fibrosis area stained with aniline blue dye (Muto Pure Chemicals Co., Ltd.) was extracted using ImageJ ver.1.41 (NIH, Bethesda, MD, USA); the fibrotic area was measured, which was divided by the interstitial area to obtain the renal fibrosis area ratio, the mean value of which was used for statistical analysis.

Alpha smooth muscle actin (α-SMA) and E-cadherin immunostaining

Deparaffinized tissue sections were washed under running water for 4 min, immersed in 10 mM sodium citrate buffer (pH 6.0) containing 0.05% Tween 20, and microwaved to activate the antigen (750 W, 5 min, four times). The sections were cooled to room temperature, washed twice with washing buffer (PBS containing 2.5% TritonX-100) for 5 min

each, immersed in blocking solution (PBS containing 3% bovine serum albumin), and stored at room temperature for 60 min. After washing with washing buffer twice for 5 min, anti-α-SMA antibody (1:1000 in blocking buffer; Abcam, Cambridge, UK) or anti-E-cadherin (24E10) rabbit mAb (1:200 in blocking buffer; Cell Signaling Technology, Danvers, USA) was added as the primary antibody, the sections were incubated at 37 °C for 60 min, and subsequently incubated at 4 °C overnight. The sections were then incubated with a goat anti-rabbit IgG H&L (1:500 with blocking buffer; Alexa Fluor® 488; Thermo Fisher Scientific K.K., Tokyo, Japan) solution as a secondary antibody, and stored at 37 °C for 60 min. Finally, the cell nuclei were stained with Hoechst 33,342 (LONZA, Walkersville, MD, USA) at room temperature for 1 min. The α-SMA-positive or E-cadherin-positive cells were examined using a fluorescence microscope (Keyence BZ-X800, Osaka, Japan), and the area of the renal cortex was measured using a BZ-X800 analyzer (Keyence, Inc.).

Statistical analysis

Data are presented as mean + standard deviation. Statistical analysis of the differences in CRE levels, BUN levels, and the ratio of renal fibrosis area and α-SMA-positive area between groups were analyzed using the Steel–Dwass test. The survival rate was analyzed using the log-rank test. $P < 0.05$ was considered statistically significant. The significance of the correlation between the renal fibrosis area ratio and CRE-, BUN-, or α-SMA-positive areas was expressed as the Spearman's rank correlation coefficient.

Results

Influence of concomitant CDDP and antihypertensive drug use on renal function markers

To confirm the effect of CDDP combined with antihypertensive drugs on nephrotoxicity, male BALB/c mice were divided into ten groups: (1) control, (2) CDDP, (3) CDDP+AML, (4) CDDP+ENA (ENA: 2.5 mg/kg), (5) CDDP+TEL (TEL: 10 mg/kg), (6) CDDP+LOS, (7) AML, (8) ENA, (9) TEL, and (10) LOS. The serum concentrations of CRE (Fig. 1a) and BUN (Fig. 1b) were measured on days −3, 0, 7, 14, 21, and 25. CDDP dosage was determined based on a previous report [17]; that is, the dosage that did not cause death or renal fibrosis during the 25-day observation period. No significant increase was observed in the CDDP+AML and CDDP+ENA groups compared to the control or CDDP groups throughout the observation period. In addition, two out of five individuals in the CDDP+AML group showed a slight decrease in CRE levels from day 7 to day 21. Although this decrease was not statistically significant, it is necessary to investigate its cause in detail. In the CDDP+LOS group, a significant increase was observed on days 7 (0.98 + 0.08 mg/dL) and 14 (0.98 + 0.15 mg/dL) compared to the CDDP group (day 7: 0.68 + 0.09 mg/dL and day 14: 0.68 + 0.09 mg/dL); however, no significant increase was observed on days 21 and 25. In the CDDP+TEL group, a significant increase was observed on day 14 (0.97 + 0.12 mg/dL) compared to the control and CDDP groups (0.68 + 0.04 mg/dL); however, no significant increase was observed on days 21 and 25. Additionally, from day 14 to day 25, BUN levels increased in all antihypertensive drug combination groups compared to the control and CDDP groups. Particularly, the CDDP+TEL group showed a significant increase in BUN levels on days 21 (144.7 + 62.2 mg/dL) and 25 (144.5 + 55.1 mg/dL) compared to the CDDP group (day 21: 29.6 + 4.8 mg/dL and day 25: 39.4 + 15.5 mg/dL) (Steel–Dwass test; $P < 0.05$).

Comparison of the survival rates of the CDDP and CDDP+ENA or CDDP+TEL groups

Of the ten mice in the CDDP+ENA group, one died on day 12, three on day 13, one on days 18, 20, 21, and two on day 24, accounting for a total of nine deaths (Fig. 2a). In the CDDP+TEL group, of the eight total mice, one died on days 5, 12, 22, and 23, accounting for a total of four mice (Fig. 2b). Meanwhile, no deaths were reported in the control, CDDP, ENA, or TEL groups, and no significant differences were observed in the survival rates of these groups compared to other groups. To investigate whether the deaths were caused by ENA and TEL doses, we examined the survival rates of the CDDP+Low ENA (ENA: 1.25 mg/kg) and CDDP+Low TEL groups (TEL: 5 mg/kg; Fig. 2a). In the CDDP+Low ENA group, 1/5 animals died on day 18. The survival rate on day 25 was 80%, which differed significantly from that of the CDDP+ENA group (10%, ENA: 2.5 mg/kg), indicating a clear dose-dependence of ENA on mortality. In the CDDP+Low TEL group, 2/5 mice died on day 6, and 1/5 died on day 22. The survival rate on day 25 was 40%, which did not differ from that of CDDP+TEL group (50%, TEL: 10 mg/kg) (log-rank test; $P < 0.05$).

Investigation of the cause of death

As described above, deaths occurred in the CDDP plus ENA and TEL groups. The cause of death was presumed to be fatal hypotension due to drug metabolism disorders and/or renal dysfunction due to CDDP accumulation. Therefore, we assessed changes in ALB, sBP, and BUN levels in each mouse (Fig. 3a). ALB level was within the reference range for all mice, suggesting that there was no hepatic dysfunction; that is, drug metabolism was not impaired. No fatal decrease in sBP level was detected immediately before death, indicating no accumulation of antihypertensive drugs. Among dead mice, four mice (44%) in the CDDP+ENA group, one (100%) in the CDDP+Low ENA group, two (50%) in the CDDP+TEL group, and one (33%) in the CDDP+Low TEL group showed elevated BUN levels immediately before death.

In the surviving mice, ALB and sBP levels were also measured, and similarly to dead mice, no hepatic dysfunction or fatal hypotension was observed (Fig. 3b). On day 25, BUN was elevated in one mouse in the CDDP+ENA group (100%), two mice in the CDDP+Low ENA group (50%), four mice in the CDDP+TEL group (100%), and one mouse in the CDDP+Low TEL group (50%), suggesting that any further administration of CDDP might be lethal due to renal injury.

Influence of concomitant use of CDDP and low-dose antihypertensive drugs on renal function markers

As described above, renal injury was the most common cause of death in mice treated with ENA and TEL. Thus, we next measured CRE (Fig. 4a) and BUN (Fig. 4b) levels during the survival period of each individual to determine whether reduced concentrations of ENA and TEL had different effects on renal damage. An increasing trend in CRE level was observed in the CDDP+Low ENA and CDDP+Low TEL groups compared to the CDDP group (day 7: 0.66 + 0.09 mg/dL, day 14: 0.68 + 0.04 mg/dL) on days 7 (CDDP+Low ENA: 0.84 + 0.17 mg/dL, CDDP+Low TEL: 1.07 + 0.15 mg/dL) and 14 (CDDP+Low ENA: 1.00 + 0.49 mg/dL, CDDP+Low TEL: 1.03 + 0.12 mg/dL). Similarly, in the CDDP+Low ENA group (day 14: 56.4 + 8.0 mg/dL, day 25: 85.8 + 6.9 mg/dL), a trend toward increased BUN level was observed compared to the CDDP group on days 14 (27.0 + 4.5 mg/dL) and 25 (39.4 + 15.5 mg/dL). The CDDP+Low TEL group showed an increase in BUN level from day 7 to day 25 compared to the CDDP group (Steel–Dwass test; $P < 0.05$).

Influence of the combination of CDDP and antihypertensive drugs on renal fibrosis

To histologically evaluate the effects of concomitant use of antihypertensive drugs on nephrotoxicity, we performed Masson trichrome staining of renal tissues and calculated the ratio of renal fibrosis area to renal interstitial area (Fig. 5a and b). Due to deaths in the CDDP+TEL, CDDP+ENA, CDDP+Low TEL, and CDDP+Low ENA groups, only

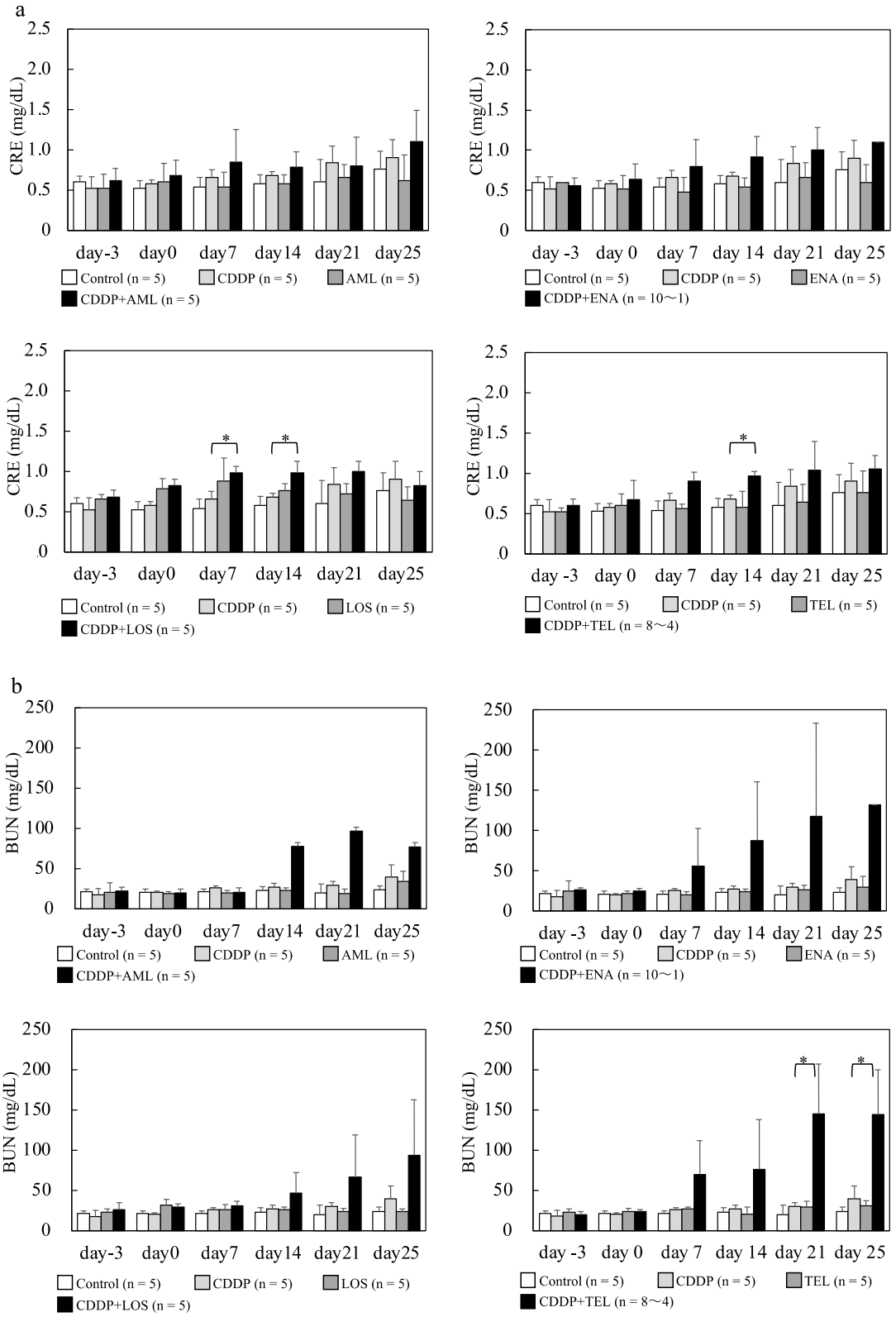

Fig. 1. Influence of different drug regimens on renal functions. BALB/c mice (6 weeks old, male) were divided into the control group ($n = 5$, untreated), CDDP group ($n = 5$, CDDP: 7 mg/kg, *i.p.*), AML group ($n = 5$, AML: 5 mg/kg), CDDP+AML group ($n = 5$, AML: 5 mg/kg), ENA group ($n = 5$, ENA: 2.5 mg/kg), CDDP+ENA group ($n = 10$, ENA: 2.5 mg/kg), LOS group ($n = 5$, LOS: 10 mg/kg), CDDP+LOS group ($n = 5$, LOS: 10 mg/kg), TEL group ($n = 5$, TEL: 10 mg/kg), and CDDP+TEL group ($n = 8$, TEL: 10 mg/kg). CDDP was administered intraperitoneally four times every 7 days (days 0, day 7, day 14, and day 21), with day 0 being the first day of administration. Serum samples were collected on days −3, 0, 7, 14, 21, and 25. (**a**) Creatinine (CRE) and (**b**) blood urea nitrogen (BUN) levels were measured in each individual, and the mean plus standard deviation (SD) was calculated. Statistically significant differences were analyzed using the Steel–Dwass test, and $P < 0.05$ was considered statistically significant (*).

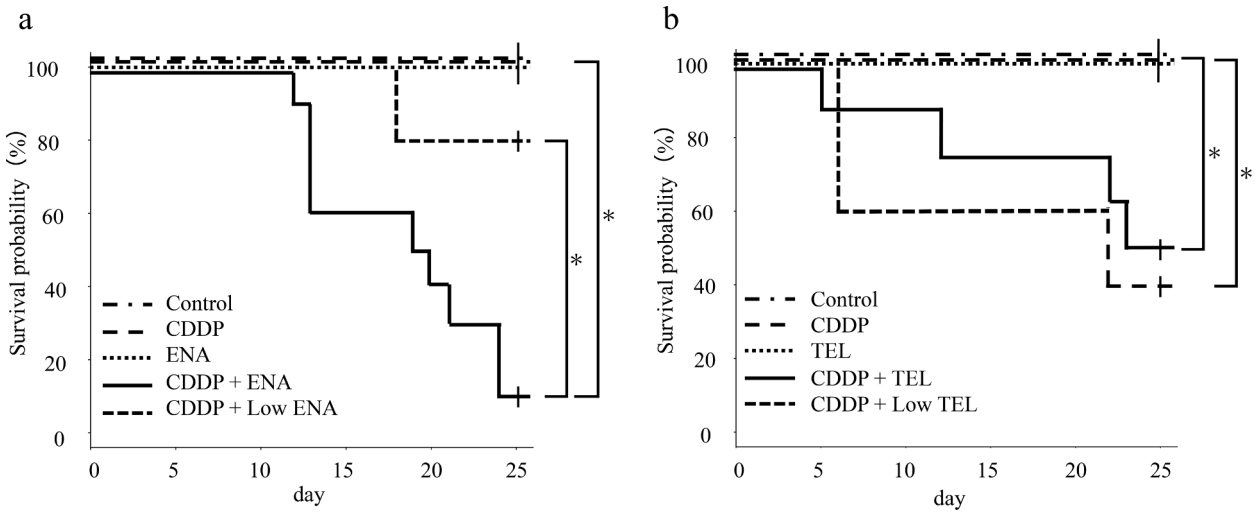

Fig. 2. Influence of CDDP combined with ENA or TEL on mouse survival. BALB/c mice (6-week-old, male) were divided into the control group ($n = 5$, untreated), CDDP group ($n = 5$, CDDP: 7 mg/kg, *i.p.*), ENA group ($n = 5$, ENA: 2.5 mg/kg), CDDP+ENA group ($n = 10$, ENA: 2.5 mg/kg), CDDP+Low ENA group ($n = 5$, ENA: 1.25 mg/kg), TEL group ($n = 5$, TEL:10 m,g/kg), CDDP+TEL group ($n = 8$, TEL:10 mg/kg), and CDDP+Low TEL group ($n = 5$, TEL:2.5 mg/kg). The first dose of CDDP was administered intraperitoneally on day 0, and drugs were administered intraperitoneally four times in seven days (days 0, 7, 14, and 21). Each antihypertensive drug was administered orally (6 times per week) from 3 days before CDDP administration (day −3) until day 24. (**a**) The survival rates of the control, CDDP, ENA, CDDP+ENA, and CDDP+Low ENA groups on day 25 were calculated. (**b**) The survival rates on day 25 were calculated for the control, TEL, CDDP, CDDP+TEL, and CDDP+Low TEL groups. Statistically significant differences were analyzed using the log-rank test, and $P < 0.05$ was considered statistically significant (*).

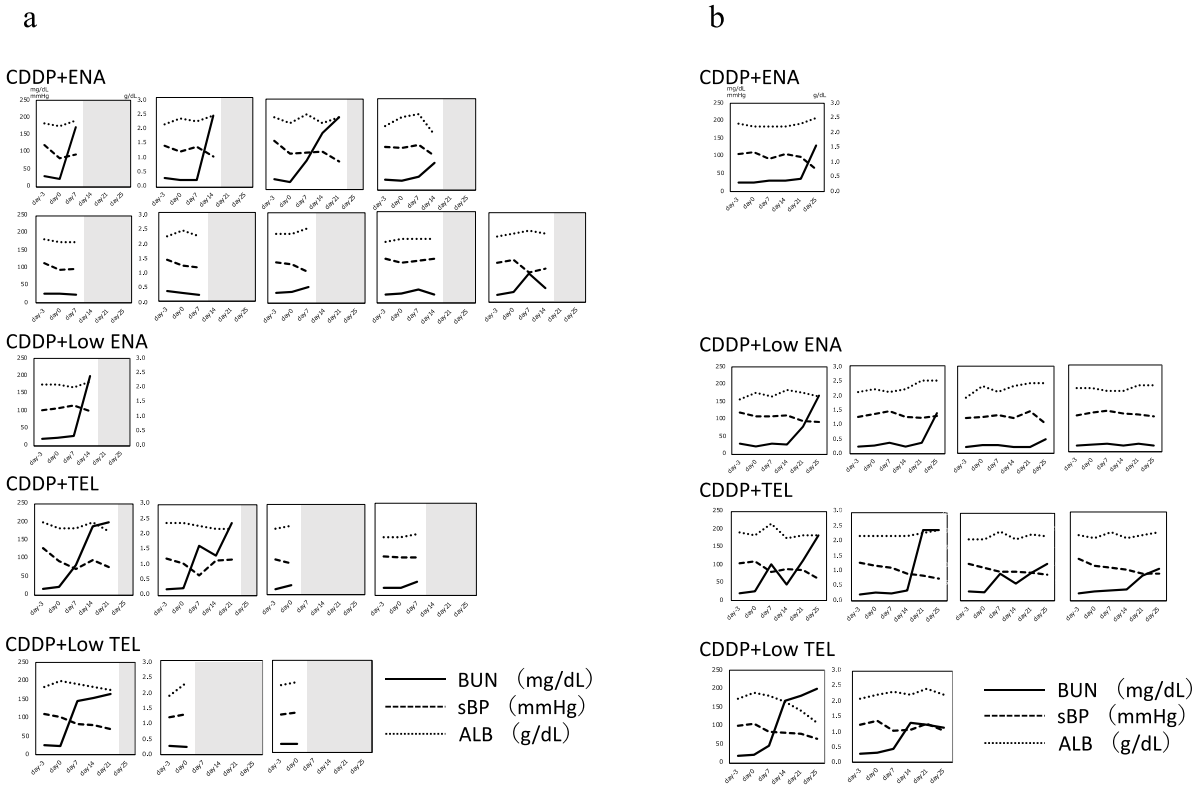

Fig. 3. Changes in blood urea nitrogen (BUN), systolic blood pressure (sBP), and serum albumin (ALB) levels in groups administered with ENA and TEL. BALB/c mice (6-week-old, male) were divided into CDDP+ENA ($n = 10$, ENA: 2.5 mg/kg), CDDP+Low ENA ($n = 5$, ENA: 1.25 mg/kg), CDDP+TEL ($n = 8$, TEL: 10 mg/kg), and CDDP+Low TEL groups ($n = 5$, TEL: 5 mg/kg). CDDP was administered intraperitoneally four times every 7 days (days 0, 7, 14 and 21), with day 0 being the first day of CDDP administration. Each antihypertensive drug was administered orally (6 times per week) from 3 days before CDDP administration (day −3) until day 24. The BUN, sBP, and ALB values of animals in each group that (**a**) died or (**b**) survived to the end of observation period are shown.

individuals who survived until day 25 were included in the analysis. The renal fibrosis area ratio was significantly increased in the CDDP group (5.7 + 0.6%) compared to the control group (1.6 + 0.7%) and each antihypertensive drug group (AML: 2.9 + 0.9%, LOS: 2.3 + 0.8%, ENA: 2.3 + 0.5% and TEL: 2.6 + 7.0%). The renal fibrosis area ratio in the CDDP+ENA (ENA: 2.5 mg/kg) (7.1%) and CDDP+TEL groups (TEL: 10

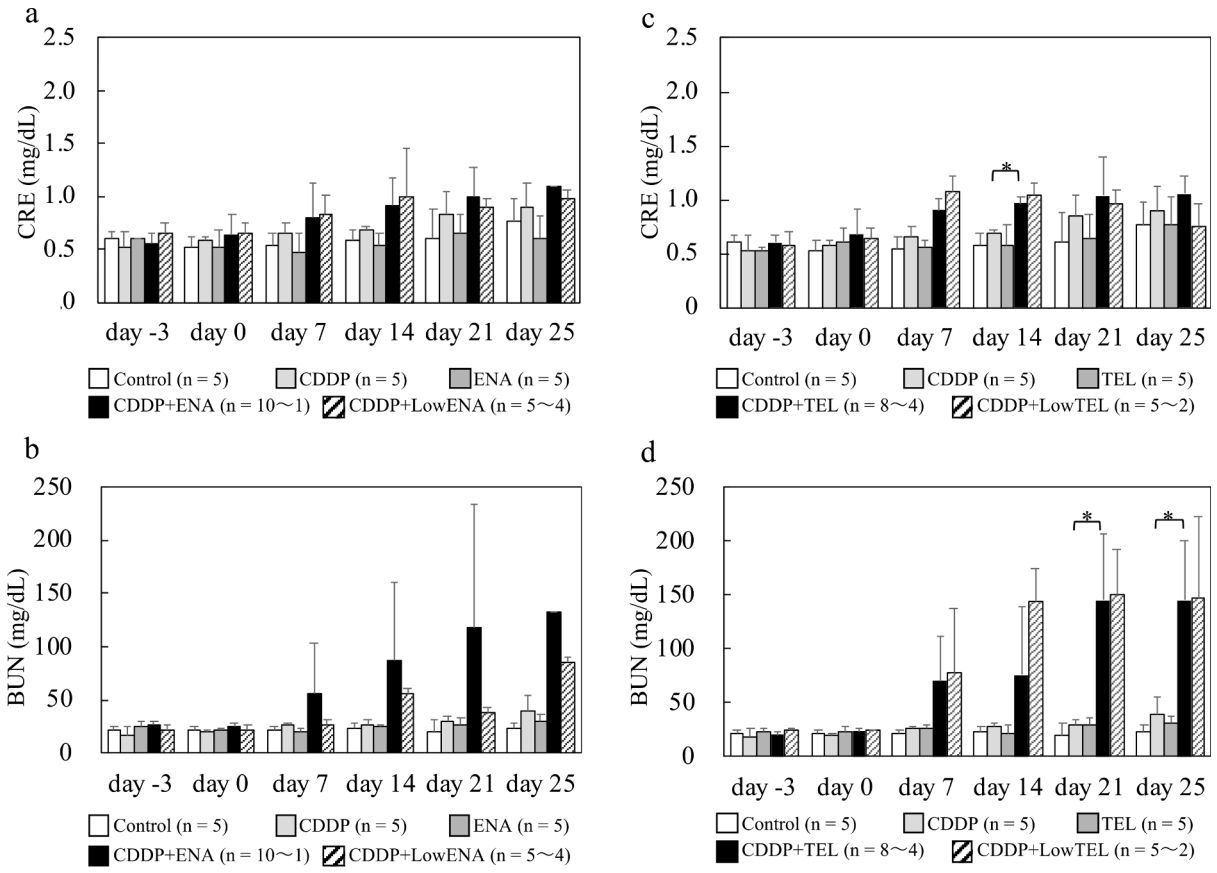

Fig. 4. Influence of the concomitant use of CDDP and antihypertensive drugs or low-dose antihypertensive drugs on renal functions. BALB/c mice (6-week-old, male) were divided into the control (n = 5, untreated), CDDP (n = 5, CDDP: 7 mg/kg, *i.p.*), ENA (n = 5, ENA: 2.5 mg/kg), CDDP+ENA (n = 10, ENA: 2.5 mg/kg), CDDP+Low ENA (n = 5, ENA: 1.25 mg/kg), TEL (n = 5, TEL:10 mg/kg), CDDP+TEL (n = 8, TEL:10 mg/kg), and CDDP+Low TEL groups (n = 5, TEL:2.5 mg/kg). The first dose of CDDP was administered intraperitoneally on day 0 for a total of four times in seven days (days 0, 7, 14, and 21). Serum samples were collected on days −3, 0, 7, 14, 21, and 25. (**a**) CRE and (**b**) BUN levels were measured in each group, and the mean plus SD was calculated. Statistically significant differences were analyzed using the Steel–Dwass test, and $P < 0.05$ was considered statistically significant (*).

mg/kg) (7.7 + 1.0%) showed an increasing trend compared to the CDDP group (5.7 + 0.6%) (Steel–Dwass test; $P < 0.05$).

Influence of concomitant CDDP and antihypertensive drug use on epithelial-mesenchymal transition (EMT)

Next, to investigate whether the combination of antihypertensive drugs induced EMT, we performed immunostaining of renal tissues for α-SMA, a marker of smooth muscle cells (Fig. 5c), and E-cadherin, a cell adhesion molecule of epithelial cells, and compared the degrees of EMT between the groups. Dead mice were excluded from the analysis. The results showed that the staining area of α-SMA tended to increase in the CDDP group (1.5 + 1.1%), as well as all CDDP plus antihypertensive drug groups (CDDP+AML: 2.0 + 1.5%, CDDP+LOS: 2.9 + 1.5%, CDDP+ENA: 5.7%, and CDDP+TEL: 4.1 + 1.6%). Particularly, the CDDP+ENA (ENA: 2.5 mg/kg) and CDDP+TEL groups (TEL: 10 mg/kg) showed a stronger tendency of increase (Fig. 5d). No such trend was observed in the E-cadherin-stained area (data not shown) (Steel–Dwass test; $P < 0.05$).

Correlation between renal fibrosis area ratio and CRE-, BUN-, or α-SMA-staining area

To investigate the reason underlying the exacerbation of renal damage with the combination of ENA and TEL, we examined the correlation between renal fibrosis area ratio and CRE or BUN levels in surviving mice (CDDP+ENA group: n = 1, CDDP+Low ENA group: n = 4, CDDP+TEL group: n = 4, CDDP+Low TEL group: n = 2). The results showed a significant correlation between the fibrosis area ratio and CRE level (r = 0.76, $P < 0.05$) with a tendency to correlate with BUN level (r = 0.46, P = 0.15; Fig. 6a and b). Additionally, a significant correlation was observed between the fibrosis area ratio and the stained area of α-SMA (r = 0.67, $P < 0.05$; Fig. 6c), thus confirming that EMT was involved in renal fibrosis (Spearman's rank correlation coefficient; $P <$ 0.05).

Discussion

In this study, we investigated the effects of repeated CDDP administration and concomitant use of antihypertensive drugs on CDDP-induced nephrotoxicity in mice. Concomitant administration of CDDP with ENA or TEL exacerbated renal damage. The concomitant use of TEL significantly increased BUN levels compared to CDDP alone, indicating that this regimen, in particular, exacerbated renal damage among the antihypertensive drugs used, which agrees with the results of a previous study [15]. Hence, concomitant use of TEL exacerbates not only CDDP-induced AKI but also chronic nephrotoxicity due to repeated administration of CDDP.

As the repeated administration of CDDP resulted in deaths in the CDDP+ENA and CDDP+TEL groups, we reduced the dose of concomitant antihypertensive drugs by half to determine whether the cause of death was dependent on antihypertensive drug dose. A significant dose dependence was observed regarding the survival rates of low and high doses of ENA, while no difference occurred in the survival rate, with no

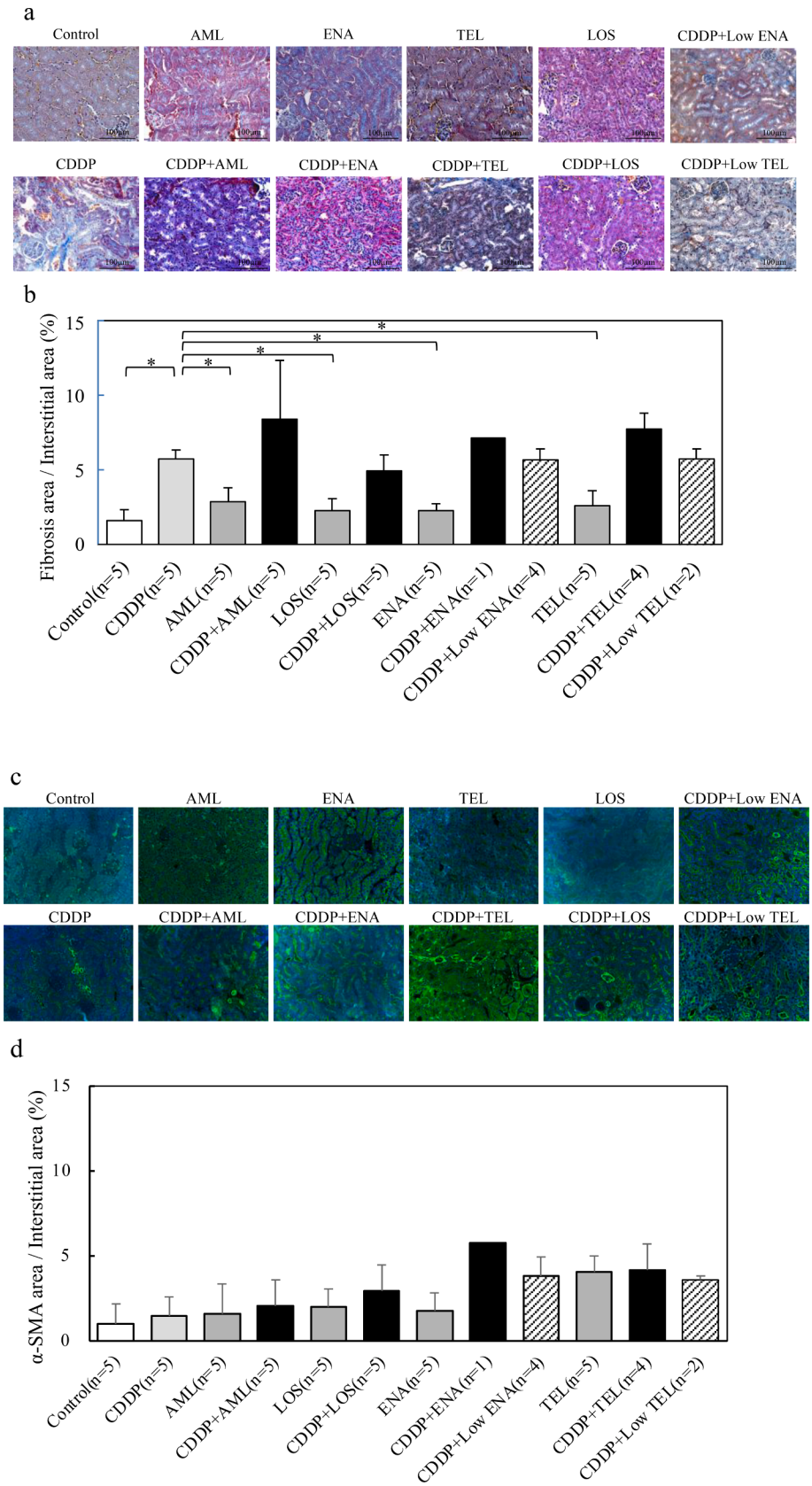

Fig. 5. Influence of CDDP in combination with antihypertensive drugs or low-dose antihypertensive drugs on renal fibrosis and epithelial-mesenchymal transition. BALB/c mice (6-week-old, male) were divided into the control ($n = 5$, untreated), CDDP ($n = 5$, CDDP: 7 mg/kg, *i.p.*), AML ($n = 5$, AML: 5 mg/kg), CDDP+AML ($n = 5$, AML: 5 mg/kg), LOS ($n = 5$, LOS: 10 mg/kg), CDDP+LOS ($n = 5$, LOS: 10 mg/kg), ENA ($n = 5$, ENA: 2.5 mg/kg), CDDP+ENA ($n = 10$, ENA: 2.5 mg/kg), CDDP+Low ENA ($n = 5$, ENA: 1.25 mg/kg), TEL ($n = 5$, TEL:10 mg/kg), CDDP+TEL ($n = 8$, TEL: 10 mg/kg), and CDDP+Low TEL groups ($n = 5$, TEL: 5 mg/kg). CDDP was administered intraperitoneally four times every 7 days (days 0, 7, 14, and 21) with day 0 being the first day of administration. Each antihypertensive drug was administered orally (6 times per week) from 3 days before CDDP administration (day −3) until day 24. (**a**) Paraffin sections were prepared from kidneys collected on day 25, and Masson trichrome staining was performed. (**b**) The ratio of fibrosis area to renal interstitial area in each group was calculated. (**c**) Another section was immunostained with an anti-α-SMA antibody. (**d**) Stained areas of α-SMA were extracted from stained images, and the ratio of stained areas of α-SMA to renal interstitial area in each group was calculated. The results of this study were analyzed in individuals who survived until the end of the observation period (day 25). The sections were examined under a microscope at a magnification of x400. Statistically significant differences were analyzed using the Steel–Dwass test, and $P < 0.05$ was considered statistically significant (*).

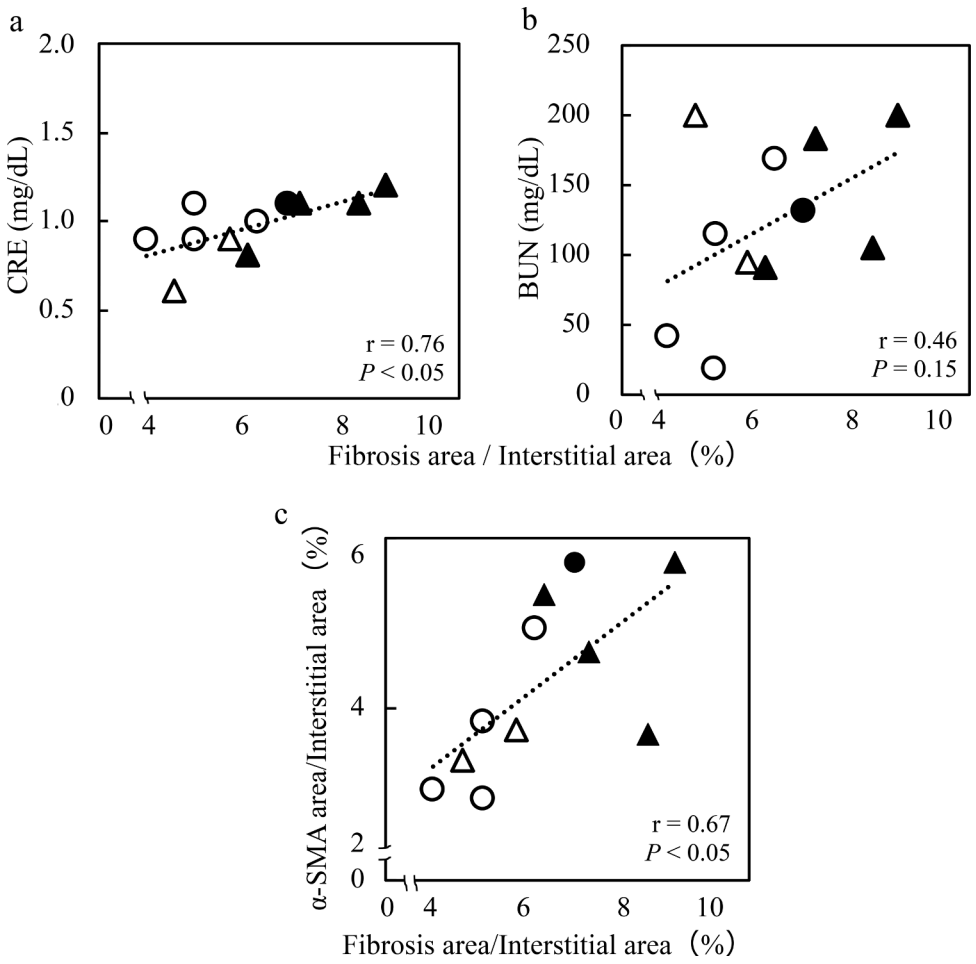

Fig. 6. Correlation between the renal fibrosis area ratio and renal function markers or stained area of α-SMA. BALB/c mice (6-week-old, male) were divided into CDDP+ENA (n = 10, ENA: 2.5 mg/kg), CDDP+Low ENA (n = 6, ENA: 1.25 mg/kg), CDDP+TEL (n = 8, TEL: 10 mg/kg), and CDDP+Low TEL groups (n = 5, TEL: 5 mg/kg). CDDP was administered intraperitoneally four times every 7 days (days 0, 7, 14 and 21) with day 0 being the first day of administration. Each antihypertensive drug was administered orally (6 times per week) from 3 days before CDDP administration (day −3) until day 24. The percentage of renal fibrosis area on day 25 was calculated and correlated with (**a**) CRE levels, (**b**) BUN levels, or (**c**) stained area of α-SMA on day 25. Statistical significance of the correlations was expressed as Spearman's rank correlation coefficient (r), where $P < 0.05$ was considered statistically significant. The results of this analysis were based on individuals who survived until the end of the observation period. CDDP+ENA, CDDP+Low ENA, CDDP+TEL, and CDDP+Low TEL groups are indicated by ○, ▲, and △, respectively.

dose-dependence, for TEL. Additionally, we examined whether death was caused by an impaired drug metabolism or lethal hypotension. ALB level was within the reference range; that is, the liver function was normal, and no lethal decrease in sBP levels of the dead mice was observed immediately before death. Therefore, hypotension due to the accumulation of antihypertensive drugs was not considered the cause of death. Of the 17 dead mice, 8 (47%) had elevated BUN levels immediately before death and were thus presumed to have died from kidney damage. Sadatomo et al. [18] reported that neutrophil-derived proteases processed macrophage-derived IL-1β precursors to the mature form and that IL-1β enhanced hepatic ischemia-reperfusion injury. In addition, Alarcon et al. [19] reported that IL-1β produced via caspase-1 after renal injury could cause arrhythmia. Therefore, the increased production of IL-1β may be a cause of death in the mice treated with CDDP and ENA or TEL in this study. This possibility needs to be investigated in detail.

ENA and TEL are reportedly taken up by the organic anion transporting polypeptides 1B1 (OATP1B1) or 1B3 in the human liver [20–24]. Moreover, CDDP is taken up by the organic cation transporter type 2 (OCT2) in the renal basement membrane, causing nephrotoxicity [25]. Meanwhile, CDDP reacts with carbonate in the blood to form negative complexes [26] and is taken up by the liver in a similar manner as TEL and ENA [27]. Therefore, TEL or ENA may have caused the dose-dependent competitive inhibition of OATP1B1 or 1B3 in the liver, resulting in enhanced uptake of CDDP by OCT2 in the renal basement membrane, exacerbation of renal injury, and death. In addition, Hye et al. [9] reported a significant decrease in renal blood flow in rats with renal dysfunction induced by CDDP administration. Therefore, it is possible that the concomitant administration of CDDP and ENA or TEL further reduced renal blood flow and exacerbated CDDP-induced renal injury, while further evidence for this relation is required.

To determine if the promotion of renal fibrosis is a mechanism by which combinatorial ENA and TEL exacerbates renal damage, we also investigated the correlation between the renal fibrosis area and renal function markers. The area of renal fibrosis correlated with CRE level and tended to correlate with BUN level, indicating that renal fibrosis reflects the pathogenesis of renal dysfunction. Additionally, the ratio of the renal fibrosis area and α-SMA-stained area were positively correlated, indicating that the induction of EMT might have promoted renal fibrosis. Moreover, the CDDP+ENA (2.5 mg/kg or 1.25 mg/kg) and CDDP+TEL groups showed an increasing trend in the α-SMA area compared to the CDDP group. Yoo et al. [28] reported that the treatment of postnatal day 7 rats with ENA increased α-SMA expression in renal tissues and significantly increased the collagen fiber area compared to the non-treated group. In the ENA group, both the staining area of α-SMA and renal fibrosis area showed an increasing trend compared to the CDDP group, which agrees with the results of a previous study [29]. Additionally, the ENA group showed an increasing trend in the stained area of α-SMA and renal fibrosis area compared to the control group. That is, the concomitant use of ENA may have induced EMT and further exacerbated CDDP-induced renal fibrosis. Meanwhile, Yadav et al. [29] reported that EMT was involved in the progression of human immunodeficiency virus-induced renal injury, and the expression of α-SMA was significantly reduced in model mice following the subcutaneous implantation of an osmotic pump filled with TEL compared to non-treated mice. This inhibitory effect of TEL on EMT is thought to result from the activation of peroxisome proliferator-activating receptor γ (PPARγ) [30]. However, these reports are not clinically relevant as they are based

on sustained TEL administration or direct stimulation of cells with TEL. In fact, Bähr et al. [31] reported that the PPARγ-activating effect of TEL was dose-dependent, and no significant difference was observed in the expression of CD163, a target of PPARγ, in humans, compared to the placebo group after administration of 80 mg or 160 mg of TEL. Furthermore, Utay et al. [32] reported that the addition of TEL to antiretroviral therapy did not inhibit adipose tissue fibrosis in patients with AIDS. Additionally, the bioavailability of the common TEL dose (40 mg) administered orally to humans is reportedly 42.4% [33]. Meanwhile, in the present study, the TEL group received 10 mg/kg of TEL orally; however, the dose was likely not sufficient to suppress EMT. In contrast to previous reports, the results of the present study indicate that TEL induces EMT. Additionally, in the TEL group, an increasing trend was observed in the stained α-SMA area; however, no similar trend was observed in the renal fibrosis area, indicating a discrepancy in results. In the future, it is necessary to subdivide the dose of the TEL group to confirm whether the induction effect of EMT is altered. Meanwhile, CDDP administration to rats reportedly increases α-SMA expression, as compared to no administration [34], and CDDP stimulates tumor-associated macrophages, thereby promoting tumor cell migration and causing EMT [35,36]. Hence, the combination of ENA or TEL may accelerate EMT induction by CDDP and exacerbate renal fibrosis.

Collectively, these results suggest that the combination of RAS inhibitors and CDDP may exacerbate renal injury. Specifically, ENA and TEL may exacerbate renal damage more strongly than other RAS inhibitors. Additionally, the EMT-induced enhancement of CDDP-induced renal fibrosis may be involved in the exacerbation of renal injury. However, we were not able to perform functional analyses in this study and are planning to conduct such experiments concurrently with our clinical studies.

Conclusions

Reduction of the therapeutic intensity of the commonly used chemotherapeutic, CDDP, interferes with the treatment of primary diseases, resulting in significantly poorer patient prognoses. Herein, we suggest that the combination of CDDP and RAS inhibitors may cause not only CDDP-induced AKI but also a decline in chronic renal function. Furthermore, no correlation was observed between CDDP-induced renal injury and hypotension, suggesting that ENA and TEL may induce EMT and promote renal fibrosis as a factor aggravating renal injury. This study provides new insights into the effect of RAS inhibitors on CDDP-induced renal injury. Hence, the basic findings of this study indicate the need for large-scale clinical studies that will aid the development of chemotherapeutic regimens that do not reduce the therapeutic intensity of CDDP nor exacerbate renal injury.

CRediT authorship contribution statement

Takumi Tsuji: Conceptualization, Methodology, Resources, Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Atsuki Hosoda:** Conceptualization, Methodology, Resources, Investigation, Formal analysis, Writing – review & editing. **Yuuki Toriyama:** Conceptualization, Methodology, Writing – review & editing. **Yuya Yoshida:** Conceptualization, Methodology, Writing – review & editing. **Takeyuki Kohno:** Conceptualization, Methodology, Writing – review & editing.

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Declaration of Competing Interest

The authors have no conflicts of interest directly relevant to the content of this article.

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