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Altered clinicopathology and renal pathology in dogs treated with a clinical dose of cisplatin

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Abstract

Cisplatin (cis-diamminedichloroplatinum, CDDP), an anti-cancer drug, might cause acute kidney injury (AKI). This study investigated clinicopathological changes in dogs treated with a clinical dose of CDDP at 0, 6, 12, 24, 72, and 168 hours. Blood testing revealed an increase of plasma IP at 6, 24, and 72 hours. Plasma Mg increased at 24 hours and decreased at 6 and 72 hours, but other parameters including Na, K, Cl, and Ca as well as blood urea nitrogen did not vary. Only plasma creatine (CRE) was elevated at 168 hours (0.68 ± 0.16 mg/dL), indicating that CDDP did not cause obvious AKI. Increases were observed, however, in urinary protein or albumin/CRE at 6 and 12 hours, urinary N-acetyl-β-D-glucosaminidase (NAG)/CRE at 24 and 72 hours, and in liver-type fatty acid binding protein/CRE at 72 hours, indicating proximal tubule (PT) injuries. Of biomarker candidates, serum and urinary miR-21, serum miR-26a and miR-10a increased. Serum miR-21 was correlated with plasma Ca, IP and Mg levels, and its urinary level correlated positively with plasma CRE and urinary NAG/CRE. Pathologically, distal tubule (DT) dilation, apoptosis in DT and PT, or *IL6*, *IL1B*, and *TNFA* renal expression tended to be elevated at 72 hours. DT dilation, *TNFA* renal expression, and macrophage infiltrations were prominent at 168 hours. Characteristically, apoptosis in the outer medulla was much greater at 72 hours than at 168 hours. We emphasize the presence of mild renal changes not clearly indicated by routine clinical examinations in CDDP-treated dogs.

Key Words: acute kidney injury (AKI), biomarkers, cisplatin nephrotoxicity, histopathology, micro RNAs (miRNA)

Introduction

In clinical veterinary medicine, dogs and cats with chronic kidney disease (CKD) are increasing in number due to increasing longevity; in particular, 30% of cats over 12 years develop CKD[36]. Companion animals with acute kidney injury (AKI) are also diagnosed in clinical cases requiring urgent care, such as poisoning as well as iatrogenic kidney disease[7]. AKI is defined in dogs as a rapid decrease of renal function within 48 hours (hr)[15]. Since mortality due to AKI is relatively high (45% and 53% in dogs and cats, respectively)[30], early diagnosis and treatment

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are crucial. As in human medicine, serum or plasma levels of creatinine (CRE) and blood urea nitrogen (BUN) are routinely used as markers of renal function. In rat model of acute kidney injury, when these parameters are increased, they represent degeneration and necrosis of tubules. This means that when these markers increased drastically, approximately 80% of renal tubular epithelial cells have damaged, and the kidneys have already suffered serious injury or may have even developed renal failure[52,65]. To overcome their low disease specificity and sensitivity[34], novel biomarkers are being developed, which would allow early and easy diagnosis. As urine can reflect the disease status of the kidney, human medicine has exploited the urinary levels of albumin (Alb), liver-type fatty acid binding protein (L-FABP), N-acetyl-ß-D-glucosaminidase (NAG), $ß_2$-microglobulin, and neutrophil gelatinase-associated lipocalin (NGAL) as renal biomarker candidates[9,16,17,19,43,44]. In veterinary medicine, the urinary Alb, NAG, and L-FABP have also been reported as potential renal biomarkers[5,51,64]. Recently, blood or urinary microRNAs (miRNAs) believed to be enveloped by exosomes have also been reported as candidate biomarkers in CKD dogs and cats[22,23]. Currently they are measured optionally but not routinely because of insufficient clinical evidence, convenience, and cost[6,46,48]. As for miRNAs, although the data in AKI is still scarce in veterinary medicine, it is suggested that these may be useful in detecting histopathological changes in kidney.

To shed light on renal pathogenesis or determine the effects of drug or chemicals, surgical treatment (e.g. unilateral ureter obstruction, nephrectomy) or administration of target substances have been performed on experimental animals[3,56]. In rodent models, anti-cancer drugs, including cisplatin (cis-diamminedichloroplatinum; CDDP), have been used to induce AKI[21]. CDDP is a platinum compound of molecular weight 300 that can be activated by removal of the leaving group and addition of H_2O via non-enzymatic process in the cytoplasm. This active CDDP subsequently induces cell death due to inhibition of deoxyribonucleic acid (DNA) synthesis by cross-linking between DNA and protein and/or DNA strands[8,59]. Based on this bioreactivity, CDDP has also been used in the treatment for solid cancers, including head and neck cancer, ovarian cancer, and osteosarcoma in humans[2,33,42]. Its dose-dependent renal toxicity causes serious problems, and approximately 30% of human patients who received CDDP developed AKI, which occasionally progressed to CKD[29,53]. CDDP is rarely used in current veterinary application for clinical treatment due to its adverse effects and the development of alternative drugs[1]. Nevertheless, CDDP has been frequently used in human medicine because the anti-tumor effect is expected. In the mouse, rat, and monkey, CDDP injured mainly the proximal tubules (PTs)[21,55], but some reports noted damage to distal tubules (DTs) and collecting ducts (CDs)[13,37,62]. In dogs, high doses of CDDP induced immediate damage to PTs, and damage to DTs was observed 48-72 hr after dosing[12]. Thus, an analysis focusing on animal-species difference, damage site, and time course in the kidney is important in evaluating drug toxicity. However, it was still unclear that the clinicopathological effect of CDDP on the dog kidney with the normal treatment-dose.

In this study, we focused here on the short-time renal toxicity of CDDP with the normal treatment-dose in dogs, and analyzed the time-course dynamics of renal functional markers as well as potential biomarkers including NAG, L-FABP and miRNAs. By combining these with renal histopathological analysis, we also discuss relations between changes in biomarkers and the injury site in the kidneys. Our data would provide further understanding of the nephrotoxicity associated with the normal treatment-dose of CDDP.

Materials and Methods

Animals and CDDP administration

All experimental protocols were approved by the animal experimental ethics committee of Osaka Prefecture University (No. 29-145, 30-149). Six female experimental dogs (beagles, 9.4 ± 1.1 kg, 1.6 ± 0.3 years) were purchased from Oriental Yeast Co., Ltd (Tokyo, Japan) and were used in this study. The dogs were diagnosed as healthy based on clinical examination including blood examination and urinalysis. They were housed individually in stainless steel cages in an air-conditioned room with a 12 hr light/dark cycle and a ventilation rate of 10-20 air changes/hr. CDDP (0.5 mg/mL, Nichi-Iko, Toyama, Japan) was administered intravenously at 70 mg/m^2 over 20 minutes (min) via the cephalic vein of dogs. This dose was based on a previous report and has been used clinically to treat osteosarcoma[40]. All samples from dogs prior to CDDP injection were defined as 0 hr sample. For histopathological analysis, two of the dogs were anesthetized deeply with propofol (6 mg/kg, i.v.) and an overdose of isoflurane (5%, i.h.), and then euthanized with an overdose of potassium chloride (4 mEq/kg, i.v.) at 72 hr and 168 hr after CDDP injection, respectively.

Blood examination and urinalysis

Blood samples were obtained from the jugular vein at 0, 6, 12, 24, 72 and 168 hr after CDDP injection. Part of the samples was used for whole blood anticoagulated with ethylenediaminetetraacetic acid (EDTA). The total red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT) levels, white blood cell (WBC) count and platelets (PLT) count in whole blood with EDTA were determined by pocH-100iV Diff (Sysmex, Kobe, Japan).

Some parts of the collected blood were mixed immediately with heparin. Sodium (Na), potassium (K), chloride (Cl) in whole blood treated with heparin were measured by on electrolyte analyzer with ion-selective electrodes SPOTCHEM EL SE-1520 (ARKRAY, Kyoto, Japan). For plasma separation, heparin-treated blood was centrifuged at 1,500 × g for 5 min at room temperature. Plasma levels of CRE, BUN, calcium (Ca), inorganic phosphorus (IP) and magnesium (Mg) were measured using an automated analyzer for clinical chemistry SPOTCHEM EZ SP-4430 (ARKRAY). For serum separation, the remaining part of the collected blood was centrifuged at 1,500 × g for 5 min after standing at room temperature for 5 min, and serum was kept at -30ºC until further analysis.

Urine samples were collected using a urethral catheter and centrifuged at 500 × g for 5 min to remove the debris. The urine samples were immediately frozen at -30ºC and stored prior to testing. Urinary levels of protein, CRE, NAG, and Alb were measured using an auto analyzer LABOSPECT 006 (Hitachi Ltd, Tokyo, Japan). Urinary L-FABP was measured with a sandwich ELISA kit according to the manufacturer's protocol (CMIC, Tokyo, Japan). The concentrations and/or activities of urinary biomarkers were normalized by the urinary CRE concentrations.

Urine protein analysis

Four-fold lithium dodecyl sulfate-sample buffer and ten-fold sample reducing reagent (Thermo Fisher Scientific, Waltham, USA) were added to the 6.5 μL of urine, and heated at 70ºC for 10 min. Electrophoresis was performed using 4-12% Bis-Tris gels and MOPS running buffer (Thermo Fisher Scientific), and 10 μL of treated solution was loaded to each lane. Gels were stained with Rapid CBB KANTO 3S according to the manufacturer's protocol (Kanto Chemical, Tokyo, Japan). Based on the size of the separated protein bands and molecular marker, the band showing approximately 65 kDa was considered as dog's Alb[54].

Histopathology

The kidneys collected were fixed in 10% neutral buffered formalin (NBF) at room temperature or 4% paraformaldehyde (PFA) at 4ºC for at least 48 hr. These fixed specimens were dehydrated using ethanol and embedded

Table 1. Primers and probes used in this study

Symbol	Sequence (5'-3')	Accession No.	Application
IL6	TCACTACCGGTCTTGTGGAGT	NM_001003301.1	RT-PCR, forward primer
	CACTCATCCTGCGACTGCAA		RT-PCR, reverse primer
IL1B	AGCAGTACCCGAACTCACCA	NM_001037971.1	RT-PCR, forward primer
	GTGGGAGACTTGCAACTGGA		RT-PCR, reverse primer
TNFA	CCTCTTGCCCAGACAGTCAA	NM_001003244.4	RT-PCR, forward primer
	TACAACCCATCTGACGGCAC		RT-PCR, reverse primer
ACTB	CAACTGGGACGACATGGAGAA	NM_001195845.2	RT-PCR, forward primer
	CATCACGATGCCAGTGGTGC		RT-PCR, reverse primer
Symbol	Sequence (5'-3')	The detail of probe	Application
miR-21	UAGCUUAUCAGACUGAUGUUGA	hsa-miR-21-5p	TaqMan PCR
		MI0000077	
miR-26a	UUCAAGUAAUCCAGGAUAGGCU	hsa-miR-26a-5p	TaqMan PCR
		MI0000083	
miR-10a	UACCCUGUAGAUCCGAAUUUGU	cfa-miR-10	TaqMan PCR
		MI0026173	
miR-10b	CCCUGUAGAACCGAAUUUGUGU	mmu-miR-10b	TaqMan PCR
		MI0000842	

RT: reverse-transcription.

in paraffin wax. Deparaffinized NBF-fixed sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), periodic acid methenamine silver (PAM), and Masson's trichrome (MT), and then renal histopathology was evaluated using these stained sections.

For immunohistochemistry, PFA-fixed paraffin sections were deparaffinized and heated with 20 mM Tris-HCl (pH 9.0) at 110°C for 15 min; the exception was a section used for single stranded DNA (ssDNA), which did not require antigen retrieval or heat. To eliminate endogenous peroxidase activity, sections were soaked in methanol containing 0.3% H_2O_2 for 20 min. The sections were then blocked by 10% normal goat serum (Nichirei, Tokyo, Japan) for 1 hr at room temperature. Next, sections were incubated overnight at 4°C with rabbit polyclonal anti-human aquaporin 1 (AQP1) antibody (1 : 50 dilution, Santa Cruz Biotechnology, Inc., Dallas, USA), rabbit polyclonal anti-human Tamm-Horsfall protein (THP) antibody (1 : 500 dilution, Senta Cruz Biotechnology, Inc.), rabbit polyclonal anti-human ionized calcium-binding adapter molecule-1 (Iba-1) antibody (1 : 1,200 dilution, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), or rabbit polyclonal anti-bovine ssDNA antibody (1 : 200 dilution, IBL, Fujioka, Japan). After washing with 0.01M phosphate buffered saline (PBS), sections were incubated with biotinylated anti-rabbit IgG antibody (Nichirei) for 30 min at room temperature. After washing with 0.01M PBS, sections were incubated with streptavidin-horseradish peroxidase conjugate (Nichirei) for 30 min at room temperature. The color was developed by incubating the sections in a solution of 3,3'-diaminobenzidine tetrahydrochloride-H_2O_2. All stained sections were converted to virtual slides using Nano Zoomer 2.0-RS (Hamamatsu Photonics, Hamamatsu, Japan).

mRNA analysis

A part of the collected kidneys was cut into

small pieces and stocked in RNAlater (Thermo Fisher Scientific) at -30ºC prior to analysis. As a control, part of a kidney from a healthy dog (HC) that had been stocked in RNAlater at -30ºC was prepared and used; when the kidney was collected, the renal function parameters as BUN and CRE were normal. Total RNA was extracted from kidney tissues using TRIzol (Thermo Fisher Scientific), and DNA digestion and reverse transcription (RT) were performed using ReverTra Ace (Toyobo, Osaka, Japan). The polymerase chain reaction (PCR) cycling was performed at 95ºC for 4 min, followed by 40 cycles of denaturation at 95ºC for 40 second (sec), annealing at 60ºC for 30 sec, and extension at 72ºC for 30 sec, using the gene-specific primer sets (Table 1) and GoTaq Green Master Mix (Promega, Madison, USA). Electrophoresis was performed using 2.0% agarose gel containing RedSafe (iNtRON Bio, Seongnam, Republic of Korea) and 0.5 × tris-borate-EDTA buffer at 100 V for 30 min. The gel was visualized under a light emitting diode and the image was captured with a digital camera.

miRNA analysis

Serum samples (200 µL) were used for total RNA preparation using a miRNeasy Serum/Plasma Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. RNA was eluted using 14 µL RNase-free water. All urine samples were centrifuged at 2,000 × g for 30 min at 4ºC to remove cells and debris. From the urine supernatant (1.0 mL), total RNA was obtained using a Urine Exosome RNA Isolation Kit (Norgen, Thorold, Canada) according to the manufacturer's instruction. All RNA solutions were adjusted to 100 µL with RNase-free water. For RT, 2.29 µL of the obtained RNA solution and 1.46 µL of reverse transcriptase solution were used with a TaqMan MicroRNA RT Kit (Thermo Fisher Scientific). From the resulting complementary DNA solution (4.5 µL), quantitative PCR analysis was performed using each miRNA-specific TaqMan primer (0.5 µL, Table 1) and TaqMan Universal PCR Master Mix (5 µL, Thermo Fisher Scientific) with a CFX Connect (Bio-Rad, Hercules, USA). Mimic miRNAs (AccuTarget; Bioneer, Daejeon, Republic of Korea) were used to draw standard curves, and the net level of miRNA was calculated using a numerical formula.

Statistical analysis

Quantitative data are expressed as the mean ± standard deviation (SD). An analysis of variance (ANOVA) test was used for comparison over three populations or time points, and multiple comparisons were performed using Dunnett's test whenever a significant difference was observed ($P < 0.05$). To examine early changes in parameters, the significant difference between 0 and 6 or 12 hr was examined using the Mann-Whitney U test ($P < 0.05$). Spearman's correlation test ($P < 0.05$) was used to analyze the correlation between two parameters.

Results

Hematological parameters in CDDP-treated dogs

The blood levels of RBC, HCT, HGB, WBC and PLT in dogs were determined by hematological analysis at 0, 6, 12, 24, 72 and 168 hr after CDDP injection (Fig. 1a-e). For RBC and HCT, there were no significant differences between time-points (Fig. 1a and b). Levels of HGB, WBC, and PLT at 168 hr were significantly lower than at 0 hr ($P < 0.05$), indicating the myelosuppression status of dogs that received CDDP one week after injection (Fig. 1c-e). In particular, WBC levels tended to decrease with time after injection (Fig. 1d).

Blood electrolytic and renal functional parameters in CDDP-treated dogs

Fig. 2 shows the blood levels of electrolytes (Fig. 2a-f) and the plasma levels of BUN and CRE measured by blood biochemistry in dogs that received (Fig. 2g and h). Na, K, Cl, and

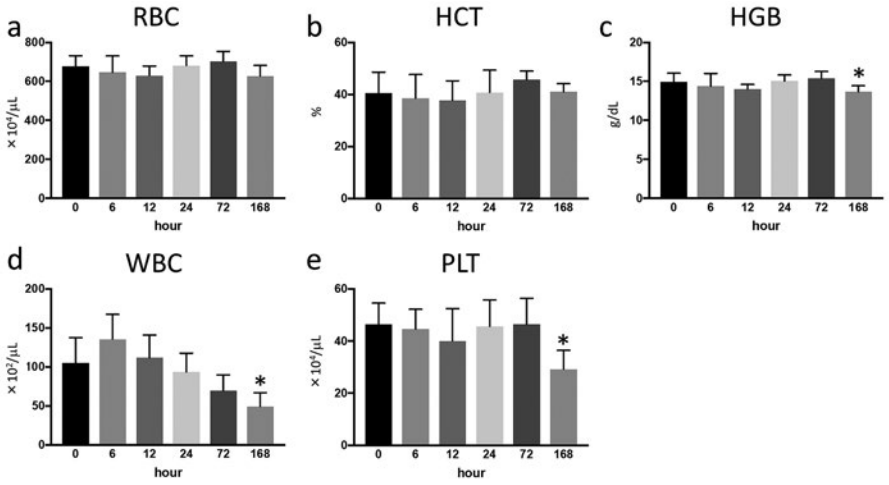

Fig. 1. Hemoanalysis in CDDP-treated dogs.
RBC (a), HCT (b), HGB (c), WBC (d) and PLT (e) in CDDP-treated dogs. Values = mean ± SD. n = 5. Any significant difference from 0 hr is indicated by * ($P < 0.05$) (Dunnett's test).

Ca underwent no significant change during the experimental periods (Fig. 2a-d). IP and Mg showed characteristic dynamics; IP was significantly raised at 6, 24 and 72 hr over 0 hr ($P < 0.05$), but Mg had decreased significantly at 6 and 72 hr and was increased at 24 hr (Fig. 2e and f) in multiple comparison tests. For early change analyzed across two groups, a significant difference between 0 hr and 6 hr was observed for IP and Mg ($P < 0.05$, Fig. 2e and f). For renal functional parameters, BUN underwent no significant change between time points (Fig. 2g), but CRE increased with time, and there was a significant difference of CRE in multiple comparison between 0 hr and 168 hr ($P < 0.05$, Fig. 2h), indicating a slight deterioration of renal function after CDDP injection at 1 week.

Urinary parameters in CDDP-treated dogs

Fig. 3 shows changes with time of urinary proteins, which altered more drastically than blood parameters examined (Fig. 1 and 2). In the electrophoresis of urine, the band with approximately 65 kDa, indicating the Alb, was increased in intensity at 6, 12, 24 and 72 hr compared to 0 hr, but at 168 hr was similar to 0 hr (Fig. 3a). Further, several bands with size below 65 kDa were also increased at 6, 12, 24 and 72 hr but not at 168 hr. For urinalysis, the urinary protein to CRE ratio (UPC) increased with time after CDDP injection until 72 hr and was decreased at 168 hr; significance in multiple comparisons was detected between 0 and 72 hr (0.16 ± 0.1 and 3.9 ± 4.1, $P < 0.05$, Fig. 3b). The urinary Alb to CRE ratio (UAC) also displayed similar dynamics to UPC, although no significant difference in multiple comparison was detected between time points (Fig. 3c). Looking at early changes analyzed by two groups testing, a significant difference between 0 hr and 6 or 12 hr was observed for UPC and UAC ($P < 0.05$, Fig. 3b and c).

Further, urinary NAG/CRE and L-FABP/CRE, which are potential renal biomarkers[5,51], showed significant increases with CDDP injection. Urinary NAG/CRE was increased with time from after CDDP injection until 72 hr and had decreased at 168 hr; significance in multiple comparison was found between 0 and 24 or 72 hr (3.1 ± 2.5 vs 17.1 ± 11.9 or 7.1 ± 5.0 U/g, $P < 0.05$, Fig. 3d). A significant increase in urinary L-FABP/CRE was observed only at 72 hr in multiple comparison (0 vs 72hr, 3.3 ± 2.3 vs 26.2 ± 20.4 μg/g, $P < 0.05$, Fig. 3e). In dogs, the diagnostic value of urinary NAG/CRE is 5.7 ± 3.4 U/g[57]. Though

Table 2. Correlations between known renal biomarkers and miRNAs in serum or urine.

	Serum				Urine			
	miR-21	miR-26a	miR-10a	miR-10b	miR-21	miR-26a	miR-10a	miR-10b
BUN	-0.273	-0.220	-0.320	0.072	-0.102	-0.177	0.090	0.095
CRE	-0.290	-0.457*	-0.407*	-0.380	0.532**	0.312	0.193	-0.009
Ca	-0.444*	-0.408*	-0.411*	-0.325	0.362	0.094	0.110	0.182
IP	0.462*	0.386	0.161	0.305	0.120	-0.141	-0.129	0.033
Mg	-0.551**	-0.526**	-0.512*	-0.077	0.177	-0.108	0.301	0.049
UPC	0.312	0.383	0.182	0.298	0.197	0.022	-0.458*	-0.556**
UAC	0.400	0.515*	0.274	0.385	0.057	-0.124	-0.518**	-0.555**
NAG/CRE	0.024	-0.015	-0.230	-0.160	0.439*	0.211	-0.024	-0.215
L-FABP/CRE	-0.042	0.005	-0.381	-0.262	-0.122	0.118	-0.050	0.150
L-FABP/CRE	-0.042	0.005	-0.381	-0.262	-0.122	0.118	-0.050	0.150

n = 4. Spearman's correlation test. *: $P < 0.05$, **$P < 0.01$. BUN: blood urea nitrogen. CRE: creatinine. Ca: calcium. IP: inorganic phosphorus. Mg: magnesium. UPC: urine protein to creatinine ratio. UAC: urine protein to creatinine ratio. NAG/CRE: N-acetyl-β-D-glucosaminidase to creatinine ratio. L-FABP/CRE: liver-type fatty acid-binding protein to creatinine ratio.

the standard value of urinary L-FABP/CRE is unclear in dogs, this is under 8.4 μg/g in human[26].

Serum and urinary miRNAs in CDDP-treated dogs

Several recent studies have reported miR-21, miR-26a, miR-10a, and miR-10b as potential urinary biomarkers in dog CKD[23]. Though the reference levels of serum miRNA are unknown, serum levels of miRNA (Fig. 4a-d) at 0 hr showed 0.2 ± 0.1 pmol, 12.5 ± 6.8 amol, 1.3 ± 0.8 amol and 0.3 ± 0.2 amol, respectively. MiR-21, miR-26a, and miR-10a tend to be increased starting 6 hr after CDDP injection, and a significant increase via multiple comparisons was detected in miR-21 (0.9 ± 0.4 pmol, Fig. 4a, $P < 0.05$). Further, all miRNAs examined tend to be increased, and miR-21, miR-26a and miR-10a increased significantly over 0 hr according to two groups tests at 6 or 12 hr (Fig. 4a-c, $P < 0.05$).

For urinary miRNAs (Fig. 4e-h), miR-21 increased gradually with time-course, with significance observed between 0 and 168 hr in multiple comparison (0 vs 168hr, 26.3 ± 3.7 vs 158.2 ± 86.7 fmol, Fig. 4e, $P < 0.05$). No significant change was detected in the other miRNAs by multiple comparison, although there was a tendency to decrease from 0 to 12 hr (Fig. 4f-h). Urinary miR-26a and miR-10b tended to increase at 72 hr (0 vs 72hr, 3.6 ± 1.1 vs 159.8 ± 226.9 amol, 0.6 ±0.4 vs 1.4 ± 2.1 amol, Fig. 4f and h). For early changes, only the levels of urinary miR-21 at 12 hr were significantly raised over 0 hr according to two groups tests ($P < 0.05$, Fig. 4e).

Table 2 summarizes the correlations between miRNA-related parameters and known renal dysfunction/injury markers. Serum miR-21 is negatively correlated with plasma Ca and Mg ($P < 0.05$, $P < 0.01$, respectively) and positively with plasma IP ($P < 0.05$). Serum miR-26a was negatively correlated with plasma CRE, Ca, and Mg ($P < 0.05$, $P < 0.05$, $P < 0.01$, respectively) and with UAC ($P < 0.05$) positively. Serum miR-10a was negatively correlated with plasma CRE, Ca and Mg ($P < 0.05$). Further, urinary miR-21 level correlated positively with plasma CRE ($P < 0.01$) and NAG/CRE ($P < 0.05$). Urinary miR-10a and miR-10b correlated negatively with UPC ($P < 0.05$, $P < 0.01$, respectively), and also with UAC ($P < 0.01$).

Renal histopathology of CDDP-treated dogs at 72 and 168 hr after injection

Renal histopathology was undertaken,

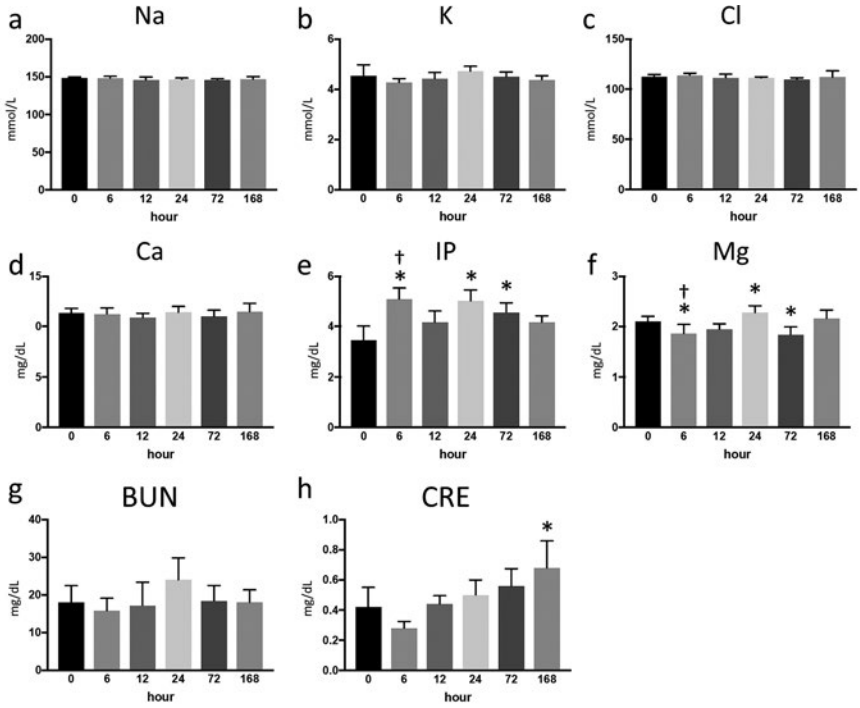

Fig. 2. Examination of blood electrolytes and renal function in CDDP-treated dogs. Na (a), K (b), Cl (c), Ca (d), IP (e), Mg (f), BUN (g) and CRE (h) in CDDP-treated dogs. Values = mean ± SD. n = 5. Any significant difference from 0 hr is indicated by * ($P < 0.05$) (Dunnett's test). A significant difference between 0 hr and 6 or 12 hr is indicated by † ($P < 0.05$) (Mann-Whitney U test).

focusing on 72 and 168 hr because a significant increase in known renal markers, such as UPC, urinary NAG/CRE, and urinary L-FABP/CRE was detected at 72 hr, and CRE at 168 hr (Fig. 2-4).

Although the sections from 72 hr after CDDP injection exhibited no remarkable histopathological changes, a few dilated tubules were observed at the renal cortex (Fig. 5a). These dilated tubules were positive for THP, which is a marker of DT segments (Fig. 5b). For immunohistochemistry of ssDNA, which prompts cell death, positive nuclei were observed in the epithelial cells in DTs as well as PTs (Fig. 5c and d) in the renal cortex; some macula densa also showed positive reactions in their nuclei. In the outer medulla, ssDNA-positive cells were detected mainly in DTs, and they tended to be observed more abundantly than in the renal cortex (Fig. 5e). These positive cells were scarcely observed in the inner medulla, however (Fig. 5f). There was no remarkable histopathological change in the glomerulus (Fig. 5g).

In contrast, the renal cortex at 168 hr after CDDP injection contained increased numbers of dilated renal tubules and PAS-positive intratubular materials, indicating protein casts (Fig. 6a and b). Further, mononuclear cells had infiltrated around these dilated tubules (Fig. 6b). As at 72 hr, these dilated tubules were positive for THP (Fig. 6c). For MT-staining, an aniline blue positive-area, indicating fibrotic regions, was observed in several regions (Fig. 6d). In the renal cortex, ssDNA-positive nuclei tended to be observed in dilated DTs; some PTs also showed a positive reaction (Fig. 6e). In the outer medulla, ssDNA-positive cells were observed in the DT, but tended to be fewer than at 72 hr (Fig. 5e and 6f). As at 72 hr, neither ssDNA-positive cells nor remarkable histopathological changes was observed in the inner medulla or the glomerulus

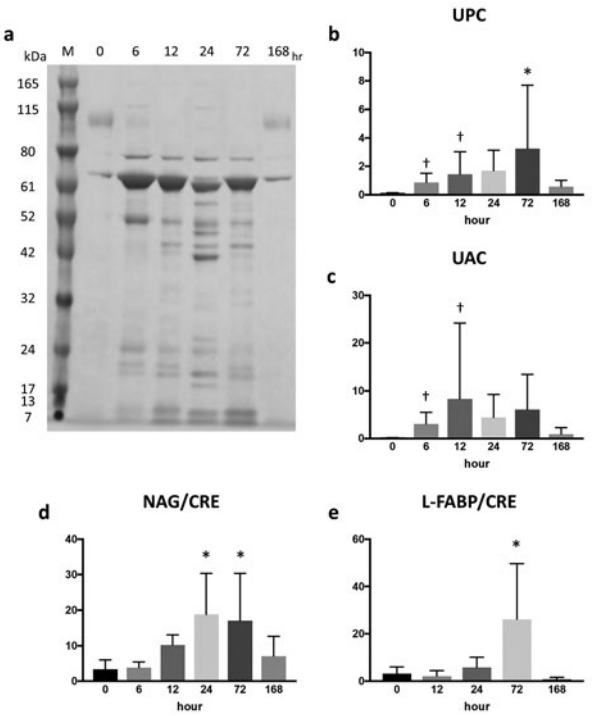

Fig. 3. Urinalysis in CDDP-treated dogs.
Electrophoresis of urine in CDDP-treated dogs (a). Arrowheads indicate the predicted size of Alb (65 kDa). M: molecular weight marker. UPC (b), UAC (c), NAG/CRE (d) and L-FABP/CRE (e) in CDDP-treated dogs. Values = mean ± SD. n = 5. Any significant difference from the 0 hr is indicated by * ($P < 0.05$) (Dunnett's test). A significant difference between 0 hr and 6 or 12 hr is indicated by † ($P < 0.05$) (Mann-Whitney U test).

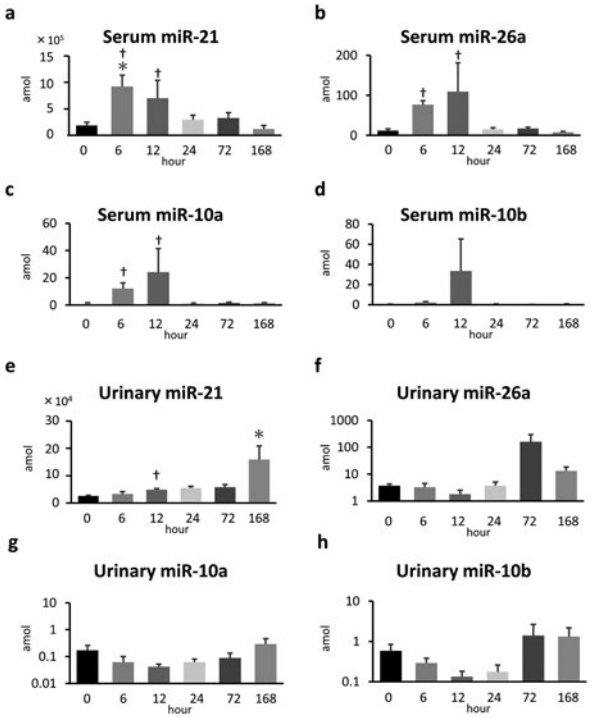

Fig. 4. Serum and urinary levels of miRNAs in CDDP-treated dogs.
miR-21 (a), miR-26a (b), miR-10a (c) and miR-10b (d) in the serum of CDDP-treated dogs. miR-21 (e), miR-26a (f), miR-10a (g) and miR-10b (h) in urine of CDDP-treated dogs. Values = mean ± SD. n = 4. Any significant difference from 0 hr is indicated by * ($P < 0.05$) (Dunnett's test). A significant difference between 0 hr and 12 hr is indicated by † ($P < 0.05$) (Mann-Whitney U test).

(Fig. 6g and h).

In the RT-PCR analysis, (Fig. 7a) specific bands of interleukin-6 (*IL6*) (amplicon size: 211 bp) and interleukin-1-beta (*IL1B*) (160 bp) were detected in kidney samples from 72 and 168 hr after CDDP treatment and HC, whereas samples from healthy control samples were faint. Further, in all samples examined, the specific band of tumor necrotizing factor-α (*TNFA*) (182 bp) was detected, and the 168 hr sample tended to give a stronger band intensity than the others. Since RT-PCR revealed a progressed inflammatory condition in CDDP-treated kidneys, we next undertook immunohistochemistry for inflammatory cells, focusing on Iba-1-positive macrophage. Iba-1-positive cells were detected in the tubulointerstitium of the kidney at 72 and 168 hr after CDDP injection, and the latter showed abundant positive cells (Fig. 7b and c).

Discussion

This study examined clinicopathological changes in dogs treated with a clinical dose of CDDP as used in osteosarcoma treatment[40]. We defined the diagnostic value in novel parameters by the data before injection CDDP, because there is little information. According to blood examination after CDDP injection, no drastic changes were observed in the parameters examined for cell counts and electrolytes. For electrolytes, the levels of IP and Mg changed significantly during 6-72 hr after CDDP injection.

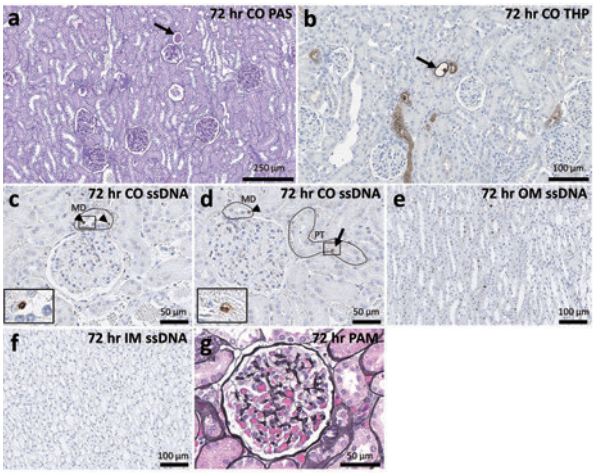

Fig. 5. Histopathological features in the kidney of CDDP-treated dogs at 72 hr.
Renal cortex (CO) stained by periodic acid Schiff (PAS) (a). No drastic alteration is observed in the glomerulus and tubulointerstitium. A few dilated tubules are observed, and occasionally contained PAS-positive protein casts (arrow). Renal CO stained by immunohistochemistry for THP, which is a distal tubule marker (b). Dilated tubule shows THP-positive reactions (arrow). Renal CO (c and d), outer medulla (OM, e), and inner medulla (IM, f) stained by immunohistochemistry for ssDNA, an apoptosis marker. ssDNA-positive nuclei are observed in the epithelial cells of the macula densa (MD, arrowheads in panels c and d) and proximal tubule (PT, arrow in panel d). Insets in panels (c) and (d) show the highly magnified area in the square area of panels (c) and (d). There are many ssDNA-positive positive cells in OM, but not in IM (e and f). Glomerular feature examined by periodic acid methenamine silver (PAM) staining (g). No proliferative or membranous alteration is observed.

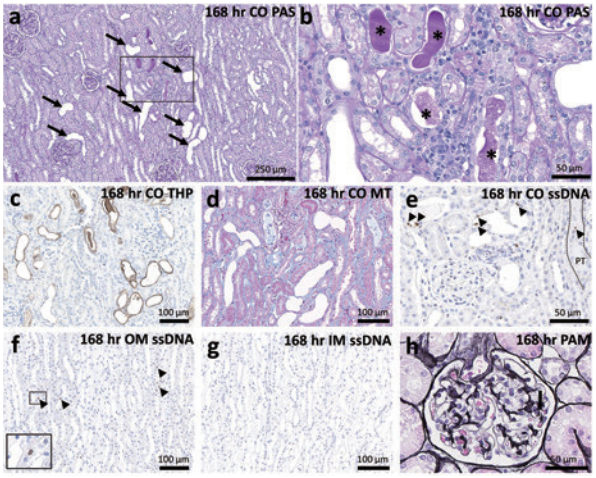

Fig. 6. Histopathological features in the kidney of CDDP-treated dogs at 168 hr.
Renal cortex (CO) stained by PAS (a and b). Several dilated tubules (arrows, panel a) and contained PAS-positive protein casts (asterisks, panel b). Panel (b) shows the highly magnified area in the square area of panels (a). Renal CO stained by immunohistochemistry for THP, a distal tubule marker (c). Many dilated tubules show THP-positive reactions. Renal CO stained by Masson's trichrome (MT, d). Aniline-blue positive fibrotic lesion is focally observed. Renal CO (e), outer medulla (OM, f), and inner medulla (IM, g) stained by immunohistochemistry for ssDNA, which is an apoptosis marker. ssDNA-positive nuclei are observed in the epithelial cells of dilated tubules and proximal tubules (arrowheads, panel e). There are ssDNA-positive positive cells in OM (arrowheads, panel f) but not in IM (g). The inset in panel (f) shows the highly magnified area in the square area of panels (f). Glomerular feature examined by periodic acid methenamine silver (PAM) staining (h). No proliferative or membranous alteration is observed.

In the dog, filtered IP and Mg were reabsorbed mainly from PT and from the ascending limb of Henle's loop (distal straight tubules), respectively[18, 47]. During 6-72 hr, although no significant change was observed in renal functional markers such as BUN and CRE, changed functioning of each nephron segment occurs after CDDP injection, as shown in the renal histopathological change at 72 hr. In fact, these CDDP-treated dogs did not satisfy the AKI diagnostic criteria defined by the International Renal Interest Society, an increase of blood CRE of more than 0.3 mg/dL within 48 hr, since plasma CRE was significantly increased only at 168 hr in our study (0 vs 168 hr, 0.42 ± 0.12 vs 0.68 ± 0.16 mg/dL). We therefore consider that the clinical dose of CDDP altered nephron function consistent with its side effects as revealed in blood electrolyte analysis; these alterations appeared earlier than those of routine markers for renal dysfunction such as BUN and CRE.

Urinary electrophoresis found an increase of Alb and several low molecular proteins after CDDP injection, and UPC and UAC changed drastically. In particular, UPC and UAC increased significantly at 6 and 12 hr after CDDP injection. In general, urinary proteins are increased due to glomerular and/or tubular injuries[10], and the contribution of the latter dominates in CDDP-treated dogs, because glomerular lesions were

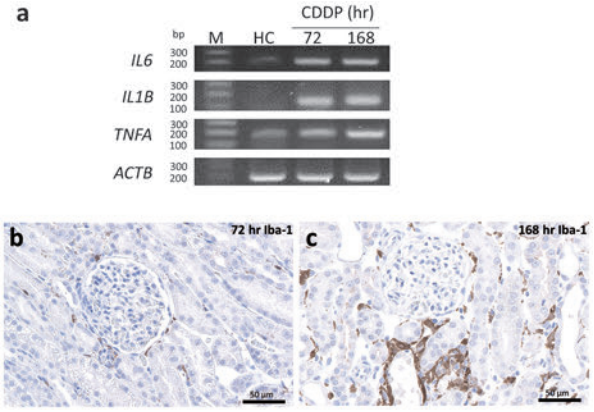

Fig. 7. Inflammatory feature in the kidney of CDDP-treated dogs at 168 hr.
RT-PCR analysis for inflammatory gene in a CDDP-treated kidney. M: size marker. HC: healthy control. The predicted bands of IL6 and IL1B are detected in the CDDP-treated kidney but not in the HC kidney. The band intensity of TNFA is stronger in the CDDP-treated kidney at 168 hr than at 72 hr and HC kidney. Renal cortex stained by immunohistochemistry for Iba-1, a macrophage marker (b and c). The Iba-1-positive cells are detected in the tubulointerstitium of the kidney at 72 and 168 hr after CDDP injection; their numbers are higher in the latter.

not obvious in this study. On the other hand, ultrastructural glomerular alterations have been reported in components of the blood urine barrier such as glomerular endothelial cells, and basement membranes, or podocytes, in CDDP-treated experimental pigs[27]. It is therefore possible that the increased UPC and UAC at 6-12 hr were caused by temporary or reversible glomerular alternations, although renal pathology was not examined in this period. In addition, urinary NAG/CRE and L-FABP/CRE increased significantly at 24-72 hr. NAG and L-FABP are respectively a deviation enzyme and an oxidative stress-associated molecule, and their increase in dog urine indicates PT injuries[63,64]. In veterinary medicine there is little information about the urinary dynamics of NAG and L-FABP in the early stages of renal damage, but the present study strongly suggests that these markers would be useful in dogs. The combination of UAC or UPC and NAG or L-FABP is therefore crucial in evaluating renal pathological changes from urine examination, and the time after injury strongly affects the dynamics of these urinary markers.

In this study, 4 miRNAs which were candidates for indicating renal injury in dogs[22] were evaluated after CDDP injection. In serum, all miRNAs examined tended to be raised after CDDP injection; only miR-21, miR-26a and miR-10a showed statistical significance at 6 and 12 hr. Further, serum miR-21 was correlated with dynamics of blood electrolytes including Ca, IP, and Mg. In addition, serum miR-21 levels correlated positively with IP but not with BUN and CRE, suggesting that miR-21 reflects the early disease status of CDDP-treated dogs. MiR-21 is expressed abundantly in the mouse kidney, but roles of miR-21 in cell differentiation and function are also suggested in the mouse blood vessels and digestive organs, which could be influenced by CDDP[35,61]. In this study, therefore, increased serum miR-21 levels might indicate kidney injury, as well as biological alerts from a damaged vascular system or other organs.

For urinary levels of miRNAs, the exosomes were collected because these contained miRNAs and this process would increase the efficiency of miRNA isolation[23]. At early time points, miR-21 was raised, but the others were reduced at 6 or 12 hr, and urinary miR-21 level was positively correlated with plasma CRE and urinary NAG/CRE. Increased urinary miR-21 is associated with the progression of renal inflammation and fibrosis in humans[58,60]. In fact, CDDP-treated dogs had renal inflammation as indicated by increased expression of *IL6*, *IL1B*, and *TNFA*, macrophage infiltrations, and local fibrotic features in the kidney at 168 hr, while urinary miR-21 was significantly increased at this point. Interestingly, urinary miR-26a and miR-10a tended to be reduced at 6 and 12 hr, similar to their urinary levels in CKD dogs[22]; these miRNAs are negatively correlated with plasma CRE. A previous study found decreased miR-26a and miR-10a in the glomerulus, but increased miR-21 in the tubulointerstitium, of CKD dog tissues[14,22]. Further, miR-26a was expressed in podocytes and

was decreased in CKD mouse glomerulus, and cultured podocytes reduced it and cytoskeleton molecules after injury[24]. Altered urinary levels of miR-21 and miR-26a (or miR-10a) might therefore reflect to tubulointerstitial and glomerular lesions by CDDP injections, respectively. In fact, urinary miR-26a levels are negatively correlated with UAC, suggesting glomerular damage in CDDP-treated dogs. On the other hand, urinary miR-26a and miR-10b tended to increase from 24 hr. CDDP therefore directly affects miRNA expressing cells, such as podocytes, in the early stage, but sustained injury might promote the release of exosomes from injured cells, as revealed in increased miR-26a levels in the supernatant of cultured podocytes after injury[28]. Taken together, altered miRNAs in body fluids reflect the disease condition of CDDP-treated dogs, and the evaluation of both serum and urine with several marker candidates is vital in their use as biomarkers.

In the present study, histopathological analysis was performed at 72 and 168 hr after CDDP injection. CDDP exhibited biphasic dynamics in dog blood, with a half-life of 22 min and 5 days in the first and second phases respectively[25]. It is therefore likely that a considerable amount of CDDP remained in the body at 72 hr, most of which was excreted via urine by 168 hr after injection. In general, renal damage to the tubulointerstitium, characterized by tubular dilation, inflammation and fibrotic features, was greater at 168 hr than 72 hr. Importantly, from the results of urinalysis, we considered that urinary protein excretions proceeded the renal histopathological deterioration but subsided by 168 hr after dosing in CDDP-treated dogs. In particular, because NAG is a deviation enzyme released from PCT epithelial cells, its excretion to urine due to cell damage was rapid as a significant increase at 24 hr. Therefore, we considered these early damages within 72 hr after CDDP injection would remain until 168 hr to develop renal histopathological changes without urinary protein alternation. In experimental animals, CDDP appears to injure the PTs, DTs, or CDs[13,21,37,55,62], and high doses of CDDP damage PTs immediately, then DTs after 48-72 hr[12]. The present dog study, using a clinical dose of CDDP, also found a stable histopathological change in the cortex, including apoptosis of PTs, at 72 and 168 hr, but no change in the inner medulla containing abundant CDs. Importantly, this study found dilation and apoptosis of DTs including macula densa in the cortex from 72 hr; and apoptosis in the outer medulla was greater at 72 hr than at 168 hr, indicating high susceptibility of DT to CDDP. DT is a crucial segment in regulating electrolytes, and the macula densa is a central system in the kidney. Further, the mRNA expression of inflammatory cytokines in the kidney tissue was greater than in HC. *IL6* is produced mainly by activated macrophages and mesenchymal cells, and its production is stimulated by *TNFA* and *IL1B*[49]. Renal tubular epithelial cells are also injured, and *TNFA* and *IL6* are released[32]. In CDDP-treated dogs, damaged tubular epithelial cells of PTs and DTs might therefore form a vicious cycle of inflammation with inflammatory cells such as Iba-1-positive macrophages[4].

In these ways, this study has elucidated the clinicopathological changes occurring in dogs treated with a clinical dose of CDDP. At an early stage after CDDP injection, changes in their function or morphology of nephron segments may be indicated by the markers, including blood levels of IP, Mg, miR-21, miR-26a, and miR-10a and urinary levels of proteins, Alb, NAG, L-FABP and miR-21; BUN and plasma CRE were not altered, however. Moreover, renal histopathology suggests that PT as well as DT was injured and underwent inflammation due to CDDP. These findings indicate that a clinical dose of CDDP does not lead to severe AKI features in dogs, but renal histopathological alterations took place according to the change of biomarker levels. This study injected CDDP to healthy dog, but CDDP was clinically applied to dogs which have tumors and age-related complications. Furthermore, in

human, the level of NAG is affected by urinary pH and circadian variation, and increases in extreme pH or in the morning[38,50], though those of L-FABP and miRNA are not clear. Therefore, the effects of age, complications, or physiological condition of animals on these biomarker dynamics should be clarified in the future study.

Recent human studies suggest that acute kidney damage is a risk factor for developing CKD in future[21]. Early examination using several biomarkers is therefore crucial in evaluating renal morpho-functional alterations by chemicals or drugs, including CDDP. In particular, the development and utilization of injury markers in DT are necessary to evaluate the renal damage adequately.

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