Protective Effect of Lactoferrin on Cisplatin-Induced Nephrotoxicity in Rats

Yumi KIMOTO^{1,2,3)}, Masaaki NISHINOHARA²⁾, Akihiko SUGIYAMA²⁾, Akihiro HARUNA³⁾ and Takashi TAKEUCHI^{2)*}

¹⁾United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi 753–8515, Japan

²⁾Course of Veterinary Laboratory Medicine, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori 680–8553, Japan

³⁾Tsuyama Animal Medical Center, Okayama 708–0806, Japan

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ABSTRACT. Although cisplatin (*cis*-diamminedichloroplatinum II) is one of the most effective chemotherapeutic agents, its clinical use is limited because of its strong side effects on the kidneys. The aim of this study was to investigate the renoprotective effect of bovine lactoferrin (bLf) in cisplatin-induced renal failure in rats. To assess the renoprotective effect of bLf, oral bLf (300 mg/kg) was administered from the day before to the fifth day after cisplatin (7 mg/kg, i.p.) injection. Daily administration of bLf histologically reduced renal tubular injury induced by cisplatin and suppressed the deterioration of renal function. Accumulated platinum content in the kidney was significantly decreased by the daily administration of bLf. Moreover, the administration of intravenous bLf caused a significant increase in urine volume in a dose-dependent manner. These results suggest that pretreatment with bLf produces a protective effect against cisplatin-induced nephrotoxicity. This protective effect of bLf involves the reduction of accumulated cisplatin in the kidney.

KEY WORDS: cisplatin, diuresis, lactoferrin, nephrotoxicity.

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Cisplatin (*cis*-diamminedichloroplatinum II) is one of the most effective chemotherapeutic agents used to treat a variety of solid tumors, but it has limited clinical application because it leads to the development of nephrotoxicity [30]. It is well known that cisplatin administered to rats damages the proximal tubule [2, 4, 14, 16, 19, 21] and that the kidney injury induced by cisplatin is similar to ischemic damage [6, 29]. The acute renal failure caused by cisplatin is typically characterized by signs such as a severe reduction in the glomerular filtration rate (GFR) [24], a variable fall in the renal blood flow [6, 29], a decrease in urinary concentrating ability and changes in urine volume and creatinine clearance [20]. It is essential to develop practical ways to counteract or minimize the side effects of cisplatin.

Lactoferrin (Lf), an iron-binding glycoprotein with a molecular weight of approximately 80 kDa, is found in milk, tears, saliva, and other external secretions, and in the secondary granules of neutrophils. Some physiological effects of Lf have been reported, including anti-inflammatory [15, 32], antinociception [9, 10], antistress [13, 26], anti-oxidant [28], cell proliferative [5] and immunoregulatory properties [1, 31]. Åbrink *et al.* [1], investigating the expression of Lf in the human kidney, showed that Lf may act as an anti-oxidant defense systems protecting the kidney against nonmicrobial oxidative injury. However, to our knowledge, there have been no published studies to date on the protec-

tive effect of Lf against nephrotoxicity.

To clarify the physiological effects of Lf, the characteristic transporting system for Lf has been investigated. Takeuchi *et al.* [27] demonstrated that intraduodenally administered bovine Lf (bLf) is transported into the blood circulation via thoracic duct lymph fluid in adult rats. Their findings indicate that the bLf transported to epithelial cells from the lumen reaches the blood circulation via the lymphatic pathway, suggesting that it could be distributed to the whole body if an effective dose was administered.

In the present study, we assessed the protective effect of bLf on cisplatin-induced nephrotoxicity in rats. We also investigated whether bLf affects the accumulation of platinum (Pt) in the kidney.

MATERIALS AND METHODS

Animals: Seven-week-old male Wistar rats were obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were maintained at a controlled temperature of $24 \pm 1^\circ\text{C}$ with a 12–12 hr light-dark cycle (light cycle, 07:00–19:00), and were given standard chow (CE-2; Nihon Clea, Tokyo, Japan). The use of these animals and the procedures performed were approved by the Animal Research Committee of Tottori University.

Chemicals: Cisplatin was purchased from Nippon Kayaku (Tokyo, Japan), and bLf was purchased from Wako Chemical Co., Ltd. (Osaka, Japan).

Blood chemistry and histological analysis of the kidney: Twenty-four rats were randomly divided into four groups of six rats each. The rats in the control group were orally administered saline (3 ml/kg) daily from the day before the study began (Day 0) to the fifth day (Day 5) of the study. Saline (14

*CORRESPONDENCE TO: TAKEUCHI, T., Course of Veterinary Laboratory Medicine, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Tottori 680–8553, Japan.
e-mail: takeuchi@muses.tottori-u.ac.jp

ml/kg, i.p.) was injected to the rat on Day 1. The rats in the bLf alone group were orally administered bLf (300 mg/kg) daily from Day 0 to Day 5, and injected with saline (14 ml/kg, i.p.) on Day 1. The rats in the cisplatin alone group were orally administered saline (3 ml/kg) daily from Days 0 to 5, and injected with cisplatin (7 mg/kg, i.p.) 2 hr after the saline administration on Day 1. This dose of cisplatin has been well documented to induce nephrotoxicity in rats [2]. The rats in the cisplatin+bLf group were orally administered bLf (300 mg/kg) daily from Days 0 to 5 and injected with cisplatin (7 mg/kg, i.p.) 2 hr after the bLf administration on Day 1. On Day 5, the rats were anesthetized with sodium pentobarbital (Somnopenyl, 50 mg/kg, i.p.; Kyoritu Pharmaceutical Co., Ltd., Tokyo, Japan), and blood samples were collected from the caudal venae cavae. These samples were transferred to heparinized tubes, and then centrifuged at 4°C for 15 min. Plasma was separated and stored at -20°C, until biochemical analyses were performed. Plasma BUN and creatinine were determined by means of a biochemical autoanalyzer (Dri-Chem 3000 V3.1-P01; Fuji Film Co., Tokyo, Japan). Both kidneys were rapidly removed, and the blood was washed away with saline. Each kidney was sliced longitudinally, and one piece from each side of the kidney was fixed in 10% neutral buffered formalin. The kidney tissues were processed for histological examination by the conventional method [25], and stained with hematoxylin and eosin (HE).

Effect of bLf on the accumulation of cisplatin in the kidney: Forty rats were divided into four groups (control, bLf alone, cisplatin alone, and cisplatin+bLf) of 10 rats each. Oral administration (saline or bLf) and intraperitoneal injection (saline or cisplatin) were the same as described above. The collection of blood and kidneys was carried out on Day 2, because it is well known that the onset of oxidation damage in the kidney is at around 48 hr after the cisplatin injection. After 48 hr of saline or cisplatin treatment, the rats were anesthetized with sodium pentobarbital (Somnopenyl, 50 mg/kg, i.p.), and blood samples were collected from the caudal venae cavae. A kidney was removed from each rat and weighed, and then stored at -20°C until the analysis of the accumulated Pt content. The kidney tissue was decomposed to mimic pyrolysis by adding nitric acid and hydrogen peroxide. Pt was measured using an inductively coupled plasma-optical emission spectrometry (ICP-OES) system (Optima 4300DV; PerkinElmer Japan, Yokohama, Japan). The range of Pt measurement was 0–500 ppb. The accumulated Pt contents in the kidney were represented as $\mu\text{g/g}$ tissue.

Diuretic effect of bLf in normal rats: Seven healthy rats were used for this experiment. Under general anesthesia with 25% urethane (1 g/kg, s.c.) and sodium pentobarbital (Somnopenyl, 25 mg/kg, i.p.), a cannula was inserted into the external jugular vein to control the depth of anesthesia by an additional injection of sodium pentobarbital. Saline (4.5 ml/kg/hr) was infused constantly by syringe pump (TOP-5300, TOP Co., Ltd., Tokyo, Japan) to maintain blood circulation and blood pressure. Another cannula was inserted into the external carotid artery to monitor blood pressure via an amplifier (PowerLab; ADInstruments, Sydney, Australia). The

heart rates of all rats were also monitored by electrocardiography (PowerLab; ADInstruments). Body temperature was monitored by a rectangular sensor (SK-1250MC III α ; Sato Keiryoki Mfg. Co., Ltd., Tokyo, Japan). During the experiment, the blood pressure of the rats was maintained within the range of 80–120 mmHg, the heart rate within 270–330 BPM, and the body temperature within 36.5–37.0°C. An abdominal midline incision was made in each rat, and the bladder and both ureters were exposed. Polyethylene tubes (SP-10; Natsume Co., Ltd., Tokyo, Japan) were placed into both ureters. BLf was administered to the rats through the external jugular vein at three doses (3, 10 and 30 mg/kg) at 40, 100 and 160 min after the start of urine sampling. During the procedures, urine samples were collected into plastic tubes every 10 min, and urine volume was measured.

Statistical analysis: All data are expressed as means $\pm$ SE. Statistical comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. A probability level of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of bLf on blood chemistry and histological findings in the kidney: Figure 1 shows the effects of bLf on the plasma concentrations of BUN (Fig. 1A) and creatinine (Fig. 1B) in normal rats and rats with acute renal failure induced by cisplatin administration. The administration of bLf alone did not change BUN or creatinine. Cisplatin caused a significant increase in BUN (14.47 ± 0.38 mg/dl in the control group and 124.32 ± 11.39 mg/dl in the cisplatin alone group) and creatinine (0.17 ± 0.02 and 2.56 ± 0.17 mg/dl, respectively). These renal abnormalities induced by cisplatin were significantly improved by pretreatment with oral bLf (the cisplatin+bLf group).

Histopathological examination of the kidney revealed that cisplatin strongly impaired proximal tubule. In the cisplatin alone group (Fig. 2C and 2E), marked tubular necrosis was observed especially in the straight portion of the proximal tubule in the outer stripe of the medulla, while bLf alone and saline groups (Fig. 2A and 2B, respectively) did not cause any pathological changes. The lesions in the cisplatin+bLf group were smaller and milder than those of cisplatin alone group (Fig. 2D and 2F). BLf administration was found to reduce the epithelial damage induced by cisplatin.

Effect of bLf on the accumulation of cisplatin in the kidney: The accumulated Pt contents in the kidney were measured at 48 hr after saline or cisplatin (7 mg/kg, i.p.) treatment (Table 1). As reported in the previous study [14], the tubular damages occurred at 48 hr after cisplatin treatment. The Pt contents in the cisplatin alone and the cisplatin+bLf groups were 16.70 ± 1.96 and 9.8 ± 2.1 $\mu\text{g/g}$ tissue, respectively (Table 1). The Pt content in the kidney was decreased significantly by bLf treatment. In this experiment, the BUN and creatinine concentrations in plasma were also measured at 48 hr after saline or cisplatin (7 mg/kg, i.p.) injection. The administration of bLf (300 mg/kg, p.o.) alone did not affect BUN or creatinine, while cisplatin caused a mild increase

Fig. 1. Effect of bLf administration on the plasma concentrations of BUN and creatinine in cisplatin-induced renal injury. Each graph shows the plasma concentrations of BUN (A) and creatinine (B) in the cisplatin-induced rats on the 5th day after the cisplatin (7 mg/kg) injection. BLf (300 mg/kg) was orally administered in the bLf alone and cisplatin+bLf groups from Day 0 to Day 5. Data represent means $\pm$ SE from 6 rats. Significant differences are shown as **, $P < 0.01$ vs. control group, §§, $P < 0.01$ vs. bLf alone group, and ##, $P < 0.01$ vs. cisplatin alone group, respectively.

in both (cisplatin alone group). These changes induced by cisplatin were significantly improved by pretreatment with oral bLf (cisplatin+bLf group).

Diuretic effect of bLf in normal rats: Intravenous bLf administration caused a significant increase in urine volume in a dose-dependent manner (Fig. 3A). Compared with the average urine volume during the first 30 min, those values after 10 and 30 mg/kg bLf administrations resulted in increments of 157 and 250%, respectively. There were no significant effects of bLf on an arterial blood pressure and a heart rate (Fig. 3B and 3C).

DISCUSSION

The present study indicated that orally administered bLf has a protective effect against the acute renal failure induced by cisplatin in rats. This protective effect of bLf was suggested by biochemical and morphological evidences, including lower BUN and creatinine levels and lower damage in the proximal tubules compared with the cisplatin alone group.

We also demonstrated that the Pt content in the kidneys

in the cisplatin+bLf group was significantly lower than that in the cisplatin alone group. Esteban-Fernandez *et al.* [7] reported that the maximum concentration of Pt in the kidney was achieved within the third day after the cisplatin treatment in rats. In our previous study, proximal tubular injury developed from the second day to the fourth day after the cisplatin injection [14]. Therefore, we measured the Pt content in the kidney at 48 hr after the cisplatin injection. Muenyi *et al.* [17] reported that the systemic Pt accumulation occurred in the following order: kidney > liver = spleen > heart > brain. It is well known the accumulated Pt contributes to the occurrence of damage to the proximal tubules. However, Esteban-Fernandez *et al.* [7] mentioned that the toxic effects produced by cisplatin in the kidney are probably not only related to the Pt accumulation and distribution, but also to the drug-biomolecule interaction, because they found that two fractions ranging below 50 kDa represented approximately 50% of the total Pt in the cytosol of the kidney. In any case, the reduction of accumulated Pt in the kidney might be related with the prevention of tubular damage induced by cisplatin.

In the present study, intravenous injection of bLf caused diuresis in a dose-dependent manner. Cisplatin causes decreased renal blood flow by renal vasoconstriction as well as ischemic damage in the proximal tubule [6, 20, 24, 29]. It is well known that several diuretic drugs such as furosemide and mannitol have a renoprotective effect in cisplatin-treated animals [18]. The increment of urine volume leads to suppression of the reabsorption of cisplatin at the proximal tubules. In our study, urine volume increased up to 2.5 times higher than the basal volume when the rat received bLf at a dose of 30 mg/kg. Hayashida *et al.* [11] reported that bLf causes a nitric oxide (NO)-dependent relaxation of smooth muscle in the rat's aorta, suggesting that bLf can affect the blood circulation in individual organs. The involvement of NO in the renoprotective effect in cisplatin-injected rats was supported by a treatment with L-arginine resulting in NO modulation [22]. Hydration with saline also has a renoprotective effect on cisplatin-injected rats [23]. In our experiment regarding the diuretic effect of bLf, the rats were infused with saline throughout the experimental period to maintain the blood circulation. Saline infusion had no significant effects on the urine volume, heart rate or blood pressure by itself. In contrast, bLf resulted in a significant increase in the urine volume without affecting the heart rate or blood pressure. Further experiments are required to clarify the precise mechanisms of the diuretic effect of bLf.

For the clinical application of bLf, the oral route may be safest, because bLf has a risk of antigenicity. Atkinson and Meredith [3] reported that bLf given through i.p. exhibited antigenicity in the Brown Norway rat model using carrageenan as an adjuvant. Ishikado *et al.* [12] also compared the oral administration and subcutaneous administration of bLf in guinea pigs in terms of antigenicity. Their report indicated that the oral administration caused less passive cutaneous anaphylaxis than the subcutaneous administration. In the present study, orally administered bLf actually indicated the protective effect on the cisplatin-induced renal failure.

Fig. 2. Histopathological findings of cisplatin-induced renal tubular injury. Bar=500 μ m (A–D). Bar=50 μ m (E and F). Photographs A–D show low magnification. Photographs E and F show high magnification. A: saline, B: bLf alone, C and E: cisplatin alone, D and F: cisplatin+bLf. Cisplatin treatment caused severe renal tubular injury in the outer stripe of the medulla (arrows) in the cisplatin alone group (C). The injured area was smaller and milder in the cisplatin+bLf group (D and F) than the cisplatin alone group (C and E).

Table 1. Effect of bLf administration on the plasma concentrations of BUN and creatinine and the Pt content in the kidney tissue in cisplatin-induced renal injury

Group	BUN (mg/dl)	Creatinine (mg/dl)	Pt content (μ g/g tissue)
Saline	14.87 $\pm$ 0.49	0.13 $\pm$ 0.02	N.D.
BLf alone	16.24 $\pm$ 0.69	0.11 $\pm$ 0.01	N.D.
Cisplatin alone	24.32 $\pm$ 1.86**	0.31 $\pm$ 0.03**	16.7 $\pm$ 1.96
Cisplatin+bLf	18.26 $\pm$ 1.66	0.15 $\pm$ 0.02##	9.8 $\pm$ 2.1#

All rats were evaluated at 48 hr after the administration of saline or cisplatin (7 mg/kg, ip). Data represent means $\pm$ SE from 10 rats. Significant differences are shown as **, $P < 0.01$ vs. saline group, ##, $P < 0.01$ vs. cisplatin alone group, and #, $P < 0.05$ vs. cisplatin alone group. N.D.: not detected.

In our previous study, orally administered Lf was detected in plasma as an intact protein [8]. Moreover, we demonstrated that intraduodenally administered bLf is transported into the blood circulation via thoracic duct lymph fluid in adult rats [27]. These characteristic transportation systems of Lf contribute to its variety of clinical effects throughout the body.

In conclusion, the present study indicates that pretreatment with bLf produces a protective effect against cisplatin-induced nephrotoxicity. It is also suggested that bLf evokes diuresis and reduces the accumulation of cisplatin in the kidney.

Fig. 3. Time course of urine output and the diuretic effect of bLf in normal rats. Each graph shows the total value of the urine (A), arterial blood pressure (B) and heart rate (C), respectively. Three doses of bLf (3, 10 and 30 mg/kg) were intravenously administered at 40, 100 and 160 min after the start of urine sampling. With respect to the arterial blood pressure (B) and heart rate (C), the effect of the drug was compared with the value measured 10 min before each drug injection. Each column shows the mean value in each 10-min interval. Data represent means $\pm$ SE from 7 rats. Significant differences are shown as *, **, corresponding to $P < 0.05$, $P < 0.01$ vs. the average volume of urine during the first 30 min, respectively.

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