Bovine lactoferrin ameliorates ferric nitrilotriacetate-induced renal oxidative damage in rats

Yasumasa Okazaki,1 Isato Kono,2 Takayoshi Kuriki,4 Satomi Funahashi,1 Soichiro Fushimi,2 Mohammad Iqbal,2,5 Shigeru Okada2,6 and Shinya Toyokuni1,*

1Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, Showa-Ku, Nagoya, Aichi 466-8550, Japan
2Department of Pathological Research and 4Department of Anti-Aging Food Sciences, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Okayama 700-8558, Japan
3Biotechnology Research Institute, University Malaysia Sabah, Locked Bag No. 2073, 88999, Kotakinabalu, Sabah, Malaysia
5Biotechnology Research Institute, University Malaysia Sabah, Locked Bag No. 2073, 88999, Kotakinabalu, Sabah, Malaysia
4Okayama Prefectual Center for Animal Husbandry & Research, Kume, Okayama 709-3401, Japan
6Biotechnology Research Institute, University Malaysia Sabah, Locked Bag No. 2073, 88999, Kotakinabalu, Sabah, Malaysia

Milk provides a well-balanced source of amino acids and other ingredients. One of the functional ingredients in milk is lactoferrin (LF). LF presents a wide variety of bioactivities and functions as a radical scavenger in models using iron-ascorbate complexes and asbestos. Human clinical trials of oral LF administration for the prevention of colon polyps have been successful and demonstrated that dietary compounds exhibit direct interactions. However, antioxidative properties of LF in distant organs require further investigation. To study the antioxidant property of LF, we employed bovine lactoferrin (bLF) using the rat model of ferric nitrilotriacetate (Fe-NTA)-induced renal tubular oxidative injury. We fed rats with bLF (0.05%, w/w) in basal chow for 4 weeks and sacrificed them after Fe-NTA treatment. After intraperitoneal administration of 9.0 mg iron/kg Fe-NTA for 4 and 24 h, bLF pretreatment suppressed elevation of serum creatinine and blood urea nitrogen levels. In addition, we observed protective effects against renal oxidative tubular damage and maintenance of antioxidant enzyme activities in the bLF-pretreated group. We thus demonstrated the antioxidative effect of bLF against Fe-NTA-induced renal oxidative injury. These results suggest that LF intake is useful for the prevention of renal tubular oxidative damage mediated by iron.

Key Words: lactoferrin, chemoprevention, ferric nitrilotriacetate, oxidative renal damage, glutathione metabolism

Life-style-related pathologic conditions, such as obesity, diabetes mellitus, hypertension, cardiovascular disease, hyperlipidemia and cancer, are causatively associated with oxidative stress. Therefore, daily dietary intake of antioxidative food may decrease the burden of oxidative stress and be beneficial for the promotion of health. During development in mammals, the neonatal stage is sensitive to oxidative insults. Dietary elements in milk might play pivotal roles in the protection of infants from harmful reactive oxygen species (ROS). Lactoferrin (LF) is one of the most functional bioavailable compounds in milk.

Previous studies have suggested that LF has beneficial effects on plasma lipids, antimicrobial, immunomodulatory, antiproliferative and antioxidant activities. LF is a member of the transferrin family of non-heme iron-binding glycoproteins and is expressed and secreted by a variety of glandular epithelial cells in adults of many species. Despite a high degree of homology at the amino acid sequence level between transferrin and LF, the function of LF differs from transferrin. Endogenous LF is induced by the exposure of iron and carcinogenic asbestos fibers in rodents, which implies a protective role for LF by forming complexes with harmful free iron in tissues in vivo. Although, LF knockout mice do not show alterations of iron homeostasis in vivo, feeding of LF-supplemented diets leads to decreased plasma triacylglycerol and free fatty acid levels and increased high-density lipoprotein levels, suggesting that LF has potential therapeutically effective functions in lifestyle-related diseases.

The iron-binding capacity of LF might mediate antioxidant activities in studies of asbestosis-induced superoxide generation in vitro. Protective effects of LF have been detected in Long-Evans Cinnamon (LEC) rats, neonates and patients with chronic hepatitis. Based on these physiological functions, LF has been evaluated in a clinical trial for metastatic renal cell carcinoma and has been used successfully in clinical trials for prevention of transformation of colon polyps. However, in vivo bioactivity of LF against iron-mediated oxidative stress is poorly understood.

In the present study, we employed an animal model of oxidative renal tubular damage that is induced by ferric nitrilotriacetate (Fe-NTA) to examine the biological effect of bovine lactoferrin (bLF), which is a common compound in our daily life. Fe-NTA, which is an iron chelate, catalyzes the generation of ROS and accelerates lipid peroxidation in the kidney through intraperitoneal (i.p.) administration in rats and mice, which revealed a high incidence of renal cell carcinoma. At the acute phase, oxidatively modified molecules are increased in the kidney after Fe-NTA treatment. Increases in malondialdehyde, thiobarbituric acid-reactive substances (TBARS), 4-hydroxy-2-nonenal (HNE)-modified proteins and 8-hydroxy-2-deoxyguanosine (8-OHdG) have been demonstrated.

Regarding genetic alternations of this carcinogenetic model, homozygous deletion of the CDKN2A/2B tumor suppressor gene, which is an inhibitor for cyclin-dependent kinase has been observed with monoallelic loss occurring as early as 3 weeks after the start of the protocol. The other target genes in carcinogenesis and progression include annexin2, piprz1, and aminoacylase1.

Well-known antioxidants such as vitamin E, curcumin, beverage-containing fermented black soybean and many other compounds have been reported to show protective effects in rodents.
this carcinogenesis model. Based on these studies, this carcinoma-
genesis model is useful for the evaluation of antioxidant properties
in the presence of excess iron in vivo. In the present study, we
disclosed for the first time that bLF has advantageous effects
on iron-induced renal injury. Possible mechanisms and their
implications will be discussed.

**Materials and Methods**

**Chemicals.** Iron nitrate enneahydrate, nitrolotriacetic acid,
oxidized and reduced glutathione (GSH), and GSH reductase
were purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan).
Nicotinamide adenine dinucleotide phosphate reduced, 5,5'-
dithio-bis-2-nitrobenzoic acid (DTNB), bovine serum albumin,
trichloroacetic acid, Tween 20 were purchased from Sigma (St.
Louis, MO). Basal MF diet (powder) was from Oriental Yeast
(Tokyo, Japan). The BCA assay kit was from Pierce (Rockford,
IL). Normal goat serum was from Vector Laboratories (Burlingame,
CA) and Histofine Simple Stain rat Max-PO (multi) was from
Nichirei (Tokyo, Japan). Liquid DAB was from DAKO (Kyoto,
Japan). Antibodies against HNE-modified proteins (HNE-J2) (23)
were purchased from Nichirei (Tokyo, Japan). The BCA assay kit
was from Pierce (Rockford, IL). Normal goat serum was from
Vector Laboratories (Burlingame, CA) and Histofine Simple Stain
rat Max-PO (multi) was from Nichirei (Tokyo, Japan). Liquid DAB
was from DAKO (Kyoto, Japan). Antibodies against HNE-modified
proteins (HNE-J2)(23) were purchased from the Japan Institute for the
Control of Aging (Shizukuika, Japan). All other chemicals were of
the highest quality available from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). bLF
was a kind gift from the Morinaga Milk Co. (Tokyo, Japan). The ingredients in bLF are summarized in Table 1.

**Preparation of Fe-NTA solution.** The Fe-NTA solution was
prepared as previously described. Antioxidative properties of bLF
were examined by using a model of acute renal injury induced by
iron overload. The experimental setup was as follows: Male Wistar rats (4
weeks old) were purchased from Japan SLC (Hamamatsu, Japan). The rats were housed in plastic
light/12-h dark cycles (room and allowed free access to distilled
water. The pH was adjusted to 7.0 with sodium bicarbonate. The
molar ratio of Fe to NTA was 1:4.

**Animal experiments.** The Animal Care Committee of
Okayama University Graduate School of Medicine and Dentistry
approved these experiments. Care and handling of the animals
were in accordance with the National Institutes of Health
Guidelines. Male Wistar rats (4 weeks old) were purchased from
Japan SLC (Hamamatsu, Japan). The rats were housed in plastic
cages of temperature-controlled (25°C with alternating 12-h
light/12-h dark cycles) room and allowed free access to distilled
water and a standard powdered-chow diet (MF) during the
experiment.

A total of 18 rats (60–80 g) were used for the following
pharmacological experiments. These rats were fed with a pow-
dered diet with different concentrations of bLF ad libitum for 4
weeks to monitor the effects on growth. In this experiment, rats
were divided into six groups (1 untreated group (n = 3) and 5 bLF
groups (1, 0.5, 0.1, 0.05 and 0.01%; n = 3)).

In the investigation of antioxidant properties of bLF, 32 rats
were randomly divided into 4 groups [untreated, bLF (0.05%) alone (n = 4), Fe-NTA alone (n = 6) and bLF (0.05%) + Fe-NTA
(n = 6)]. These rats were fed with the regular powdered diet or
powdered diet with bLF (0.05%, w/w) ad libitum for 4 weeks.
Each animal received an i.p. injection of Fe-NTA (9.0 mg
iron/kg body weight). After sacrificing the animals, the sera
were harvested, and the kidneys were immediately excised for
enzyme assays and histological examination.

**Determination of creatinine, blood urea nitrogen (BUN),
and serum iron-binding capacity.** Blood urea nitrogen, creat-
inine, unsaturated iron-binding capacity (UIBC) and total iron-
binding capacity (TIBC) in sera were measured using an auto-
analyzer (Hitachi 7600-110S).

**Determination of reduced GSH and antioxidant enzyme
activities of kidney.** These enzymatic activities were assayed
with the post-mitochondrial supernatant of homogenized renal
tissue. Reduced GSH levels were measured using DTNB as a
chromogen.(15) GSH peroxidase and GSH reductase activities
were measured based on NADPH oxidation in a coupled system.(35,36)

**Hematoxylin and eosin (HE) staining.** Kidneys were trans-
versely cut including the renal pelvis at 5-mm thickness and
immediately fixed with 10% phosphatase-buffered formalin.
The samples were fixed overnight and subjected to paraffin
embedding. The paraffin-embedded tissues were sliced into 4-μm sections
and mounted onto glass slides. These slides were used for
hematoxylin and eosin staining and immunohistochemical analyses.

**Immunohistochemical analysis.** Immunohistochemical
analyses were performed as previously described.(24,32,37)

**Statistical analysis.** Statistical analyses were performed
using one-way analysis of variance (ANOVA) and an unpaired
t test. The difference was considered significant when p<0.05.
The data were expressed as the mean ± SEM (n = 3–6) unless
otherwise specified.

**Results**

**The effect of bLF on growth and serum iron-binding
capacity.** We observed no growth retardation during bLF
feeding (1, 0.5, 0.1, 0.05 and 0.01%; w/w) (Fig. 1). After 4 weeks,
rats received i.p. treatment of Fe-NTA (9.0 mg iron/kg body
weight). We observed that 0.05% (w/w) bLF was the most
efficient to protect kidney from acute tubular injury compared to
other doses of bLF (data not shown). Based on these preliminary
experiments, we performed the following experiments with 0.05%
(w/w) bLF. Iron levels, total iron-binding capacity (TIBC) and
unsaturated iron-binding capacity (UIBC) were not altered after
treatment of bLF (0.05%, w/w) in rats (transferrin saturation of
untreated control, 24.7 ± 2.54%; transferrin saturation of bLF
alone, 22.6 ± 0.69%; n = 4; p = 0.44, untreated vs bLF alone).

### Table 1. Ingredients of bovine lactoferrin (bLF)

| Ingredient            | Value          |
|-----------------------|----------------|
| Calorie               | 4 kcal/g       |
| Carbohydrate          | 0%             |
| Protein               | 99.8%          |
| Fat                   | <0.5%          |
| Purity of bLF         | 96.7%          |
| Ash                   | 190 mg/100 g   |
| Sodium                | 54.7 mg/100 g  |
| Iron                  | 8.60 mg/100 g  |
| Calcium               | 8.54 mg/100 g  |
| Phosphorus            | 2.21 mg/100 g  |
| Potassium             | 1.94 mg/100 g  |
| Magnesium             | 0.58 mg/100 g  |
| Zinc                  | 0.27 mg/100 g  |

**Fig. 1.** The effect of bovine lactoferrin (bLF) on normal growth. Body
weight curve. All rats were active and healthy following administration
of different concentrations of bLF (1, 0.5, 0.1, 0.05 and 0.01% w/w)
(n = 3).
Serum creatinine and BUN. After Fe-NTA administration for 4 and 24 h, an elevation of creatinine and BUN in sera was detected. Pretreatment with bLF suppressed the elevations of these parameters at both time points (Fig. 2 A–D). A similar effect was observed in creatinine and BUN levels.

Determination of reduced GSH. The pretreatment with bLF induced slight elevations of renal reduced GSH without statistical significance ($p = 0.25$). GSH was decreased 4 h after Fe-NTA administration without bLF pretreatment. These alterations were significantly suppressed by bLF pretreatment (Fig. 3A).

Antioxidative enzyme activity. After Fe-NTA treatment for 4 h, activities of the GSH peroxidase and GSH reductase were decreased, which was prevented by bLF pretreatment. However, no elevation of these enzymatic activities was detected following bLF pretreatment. Levels of GSH reductase and GSH peroxidase were maintained in the bLF-pretreated group during oxidative injury at 24 h after Fe-NTA treatment (Fig. 3 B and C).

Histology. We evaluated the renal histology of Fe-NTA-treated rats 4 h after Fe-NTA treatment (Fig. 4 A–F). Acute tubular necrosis was apparent in the proximal tubules, after Fe-NTA treatment. Pretreated rats with bLF were protected from tubular injury (Fig. 4C).

In the cases of Fe-NTA-treated rats, degenerative proximal tubular cells with HNE-modified proteins were observed. However, the number of HNE-positive tubules was significantly decreased in the bLF-treated group. Positive tubules with apparent HNE-modified proteins were not observed in the untreated group (Fig. 4 D–F).

We evaluated the renal histology of Fe-NTA-treated rats 24 h after Fe-NTA treatment. Massive destruction of proximal tubules and cast depositions were observed in the Fe-NTA-treated group (Fig. 4G). These alternations were confined to localized areas by bLF pretreatment (Fig. 4H). Few infiltrating inflammatory cells were observed in the kidney after Fe-NTA treatment.

Discussion

Fe-NTA causes renal cell carcinoma via iron-mediated oxidative stress in rats, with homozygous deletion of $\text{CDKN2A}/\text{2B}$ in one-third of the cases. (25,26) This is a unique experimental model, useful in search of antioxidant compounds by the pretreatment for 4 weeks and of chemopreventive compounds by the
dietary administration for several months.\(^\text{31-33}\) We observed that dietary administration of \(\text{bLF (0.05\%, w/w)}\) protected renal tissue from Fe-NTA-induced damage. We demonstrated that \(\text{bLF}\) inhibited elevation of serum markers of acute renal failure and renal tubular injury. In addition, \(\text{bLF}\) pretreatment maintained the levels of reduced GSH, GSH peroxidase and GSH reductase activities. Previous studies have demonstrated that \(\text{bLF}\) pretreatment induces antioxidative enzymes in a colon cancer cell line.\(^\text{38}\) We observed that dietary intake of \(\text{bLF}\) increased GSH and GSH reductase levels. However, this increase was not statistically significant \((p = 0.25\) and \(p = 0.55\) for GSH and GSH reductase, respectively, untreated vs \(\text{bLF}\) alone).

In this study, we employed regular bovine lactoferrin (\(\text{bLF}\)), which contains apo-LF and holo-LF. Consistent with the results of previous studies,\(^\text{39}\) no toxic effects of \(\text{bLF (1\%, w/w)}\) were observed based on growth and BUN and creatine levels. In addition, we did not detect significant changes in TIBC, UIBC and transferrin saturation after \(\text{bLF}\) treatment \((0.05\%, \text{w/w})\). In our experimental design, rats ate 20–25 g/day, which was used to estimate \(\text{bLF}\) intake as 10–12.5 mg/day. This calculation suggested that intact \(\text{bLF}\) did not enter blood stream to increase the levels of iron saturation in transferrin, which was confirmed using the conventional chemical detection method in this study and is consistent with previous reports.\(^\text{10,40}\) Non-transferrin-bound iron (NTBI) in the sera is an emerging marker of iron toxicity.\(^\text{41,42}\) Although we did not measure NTBI levels in the sera, quantification of NTBI might disclose evidence as a role of \(\text{bLF}\) in antioxidant modulation via chelation of Fe-NTA.

Detection of LF in the urine suggested that LF might directly interact with proximal tubules.\(^\text{6}\) We have not determined the endogenous rat LF in the kidney. However, a possible mechanism of altered endogenous LF levels may reveal concomitant alterations in the GSH cycle in renal proximal tubules, which is directly associated with the pathologic mechanism of Fe-NTA toxicity.\(^\text{18,33}\) Other possible mechanisms of LF-mediated attenuation of Fe-NTA-induced oxidative stress include LF-mediated effects on

---

**Fig. 3.** Determination of renal reduced glutathione (GSH) at 4 h and of activities of renal antioxidative enzymes at 4 and 24 h after Fe-NTA administration. (A) GSH: Fe-NTA, 4 h. (B) GSH reductase: Fe-NTA, 4 and 24 h. (C) GSH peroxidase: Fe-NTA, 4 and 24 h. (A) Bovine lactoferrin (\(\text{bLF, 0.05\%, w/w}\)) pretreatment demonstrated slight elevation of renal GSH level \((p = 0.25,\) untreated vs \(\text{bLF alone})\). GSH depletion was detected in Fe-NTA-administered rats. We observed that \(\text{bLF}\) pretreatment attenuated Fe-NTA-induced renal oxidative damage. (B) and (C) Protective effect of \(\text{bLF (0.05\%, w/w)}\) pretreatment on Fe-NTA-induced renal oxidative damage was observed based on both parameters (ANOVA, \(p < 0.0001,\) for A-C; *\(p < 0.05\) vs untreated or \(\text{bLF alone; *p < 0.05 and **p < 0.01 vs Fe-NTA alone)\).
serum NTBI levels and Fe-NTA absorption from the peritoneum. These proposed mechanisms require further investigation.

Previous studies have revealed chemopreventive activity of bLF outside the digestive tract, such as hepatitis in LEC rats, lung tumors and chronic hepatitis. In LEC rats, LF improves rat survival and inhibits mitochondrial 8-OHdG formation but not nuclear 8-OHdG. These results indicate that LF suppresses copper-induced oxidative stress. In female Ogg1 knockout mice, bLF suppresses lung tumor development produced via 8-OHdG accumulation. These results imply that dietary intake of bLF has antioxidative effects.

Recent clinical trials have tested the effects of dietary intake of bLF on metastatic renal cell carcinoma. The mechanism of anti-tumor bioactivity was suggested that bLF may stimulate production of Interferon-gamma (IFN-γ) and IL-12, which promote a Th1 response in the intestinal mucosa. This polarization of Th1-activated neutrophils, macrophages, NK cells and cytotoxic T cells enhances anti-tumor activities and may contribute to the protection of renal oxidative injury. Activation of the IL-12/IFN-γ signaling pathway is required in ischemia-reperfusion injury, where harmful ROS play a critical role in mice.

In our model of renal cell carcinoma, which is positive for transforming growth factor-α, we observed few infiltrating inflammatory cells after a single dose of Fe-NTA (Fig. 4). We previously demonstrated that iron loading by iron saccharate enhances the production of systemic inflammatory cytokines following lipopolysaccharide stimulation. However, interactions between Fe-NTA-induced renal damage and cytokines requires further investigation.

As another possible mechanism, digested peptides that are derived from bLF or secreted from the gastrointestinal mucosa following activation of immune and neuroendocrine systems might play synergistic roles to protect the kidney from iron-induced oxidative stress. A profiling analysis using microarray techniques might elucidate bLF-interacting molecules after oral administration of bLF.

In conclusion, we observed, for the first time, that bLF had protective effects against iron-induced renal tubular injury in rats. Further study is warranted to disclose human pathologic conditions or genotypes where this kind of chemoprevention is useful.

Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Grant-in-Aid for Young Scientists (B) (23790440) from the Japan Society for the Promotion of Science and the grant from Bioactive Okayama. The authors wish to thank Morinaga Milk Co. for the kind gift and chemical analysis of bLF, Hideo Sakai and Yoko Watanabe for animal care, Emiko Okazaki for technical assistance and Masayoshi Fujisawa (Himeji Red Cross Hospital) for discussion.
Abbreviations

ANOVA analysis of variance
bLf bovine lactoferrin
BUN blood urea nitrogen
dAB 3,3'-diaminobenzidine
DTNB 5,5'-dithio-bis-2-nitrobenzoic acid
Fe-NTA ferric nitrilotriacetate
GSH glutathione
HNE 4-hydroxy-2-nonalen
IFN-γ Interferon-gamma
IL-12 interleukin-12
i.p. intraperitoneal
LEC Long-Evans Cinnamon
LF lactoferrin
NTBI non-transferrin-bound iron
8-OHdG 8-hydroxy-2'-deoxyguanosine
ROS reactive oxygen species

References

1 Toyokuni S. Iron as a target of chemoprevention for longevity in humans. Free Radic Res 2011; 45: 906–917.
2 Diplock AT, Charleux JL, Crozier-Willi G, et al. Functional food science and defence against reactive oxidative species. Br J Nutr 1998; 80 (Suppl 1): S77–S112.
3 Raghuvir SE, McGuire EM, Martin SM, et al. Lactoferrin in the preterm infants’ diet attenuates iron-induced oxidative products. Pediatr Res 2002; 52: 964–972.
4 Lönnerdal B. Nutritional and physiologic significance of human milk proteins. Am J Clin Nutr 2003; 77: 1537S–1543S.
5 Aisen P, Leibman A. Lactoferrin and transferrin: a comparative study. Biochim Biophys Acta 1972; 257: 314–323.
6 Levaey PF, Vrijmoet M. Lactoferrin: a general review. Haematologia 1995; 80: 252-267.
7 Metz-Boutigue MH, Jollès J, Mazauric I, et al. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. Eur J Biochem 1984; 145: 659–676.
8 Ghio AJ, Carter JD, Richards JH, Brighten LE, Lay JC, Devlin RB. Disruption of normal iron homeostasis after bronchial instillation of an iron-containing particle. Am J Physiol 1998; 274: L396–L403.
9 Ghio AJ, Stonehuerner J, Richards JH, Devlin RB. Iron homeostasis in the lung following asbestos exposure. Antioxid Redox Signal 2008; 10: 371–377.
10 Ward PP, Mendoza-Meneses M, Cunningham GA, Connoly OM. Iron status in mice carrying a targeted disruption of lactoferrin. Mol Cell Biol 2003; 23: 178–185.
11 Takeuchi T, Shimizu H, Ando K, Harada E. Bovine lactoferrin reduces plasma triacylglycerol and NEFA accompanied by decreased hepatic cholesterol and triacylglycerol contents in rodents. Br J Nutr 2004; 91: 533–538.
12 Ghio AJ, Stonehuerner J, Steele MP, Cumblish AL. Phagocyte-generated superoxide reduces Fe2+ to displace it from the surface of asessos. Arch Biochem Biophys 1994; 315: 219–225.
13 Tsubota A, Yoshikawa T, Nariai K, et al. Bovine lactoferrin potently inhibits liver mitochondrial 8-OHdG levels and retrieves hepatic OGG1 activities in Long-Evans Cinnamon rats. J Hepatol 2008; 48: 486–493.
14 Konishi M, Iwasa M, Yamauchi K, et al. Lactoferrin inhibits lipid peroxidation in patients with chronic hepatitis C. J Hepatol 2006; 44: 378–383.
15 Jonasch E, Stadler WM, Bukowski RM, et al. Phase 2 trial of talcraferrin in previously treated patients with metastatic renal cell carcinoma. Cancer 2008; 113: 72–77.
16 Tsuda H, Kozu T, Iinuma G, et al. Cancer prevention by bovine lactoferrin: from animal studies to human trial. Biometals 2010; 23: 399–409.
17 Hamazaki S, Okada S, Ebina Y, Midorikawa O. Acute renal failure and glucosuria induced by ferric nitroltriacetaete in rats. Toxicol Appl Pharmacol 1985; 77: 267–274.
18 Hamazaki S, Okada S, Toyokuni S, Midorikawa O. Thiobarbituric acid-reactive substance formation of rat kidney brush border membrane vesicles induced by ferric nitriltriacetaete. Arch Biochem Biophys 1985; 247: 348–354.
19 Toyokuni S. Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 1999; 49: 91–102.
20 Uchida K, Fukuda A, Kawakishi S, Hiai H, Toyokuni S. A renal carcinoma ferric nitriltriacetaete mediates a temporary accumulation of aldehyde-modified proteins within cytosolic compartment of rat kidney. Arch Biochem Biophys 1995; 317: 405–411.
21 Toyokuni S, Uchida K, Okamoto K, Hattori-Nakakuki Y, Hiai H, Stadtlm ER. Formation of 4-hydroxy-2-nonalen-modified proteins in the renal proximal tubules of rats treated with a renal carcinogen, ferric nitriltriacetaete. Proc Natl Acad Sci USA 1994; 91: 2616–2620.
22 Toyokuni S, Zhao XP, Tanaka T, Uchida K, Hiai H, Lehotay DC. Induction of a wide range of C2-12 aldehydes and C2-12 acyloins in the kidney of Wistar rats after treatment with a renal carcinogen, ferric nitriltriacetaete. Free Radic Biol Med 1997; 22: 1019–1027.
23 Toyokuni S, Miyake N, Hiai H, et al. The monoclonal antibody specific for the 4-hydroxy-2-nonalen histidine adduct. FEBS Lett 1995; 359: 189–191.
24 Toyokuni S, Tanaka T, Hattori Y, et al. Quantitative immunohistochemical determination of 4-hydroxy-2-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitriltriacetaete-induced renal carcinogenesis model. Lab Invest 1997; 76: 365–374.
25 Tanaka T, Iwasa Y, Kondo S, Hiai H, Toyokuni S. High incidence of allleic loss on chromosome 5 and inactivation of p15INK4A and p16INK4A tumor suppressor genes in oxystress-induced renal cell carcinoma of rats. Oncogene 1999; 18: 3793–3797.
26 Hiroysu M, Ozeki M, Kohda H, et al. Specific allelic loss of p15INK4A tumor suppressor gene after weeks of iron-mediated oxidative damage during rat renal carcinogenesis. Am J Pathol 2002; 160: 419–424.
27 Tanaka T, Akatsuka S, Ozeki M, Shirase T, Hiai H, Toyokuni S. Redox regulation of annexin 2 and its implications for oxidative stress-induced renal carcinogenesis and metastasis. Oncogene 2004; 23: 3980–3989.
28 Liu YT, Shang D, Akatsuka S, et al. Chronic oxidative stress causes amplification and overexpression of p16INK4A protein inactivation by age-related decline in p53 function. Carcinogenesis 2004; 25: 939–948.
29 Zhong Y, Onuki J, Yamasaki T, Ogawa O, Akatsuka S, Toyokuni S. Genome-wide analysis identifies a tumor suppressor role for a microRNA-27a in iron-induced rat renal cell carcinoma. Carcinogenesis 2009; 30: 158–164.
30 Zhang D, Okada S, Yu Y, Zheng P, Yamaguchi R, Kasai H. Vitamin E inhibits apoptosis, DNA modification, and cancer incidence induced by iron-mediated peroxidation in Wistar rat kidney. Cancer Res 1997; 57: 2410–2414.
31 Okazaki Y, Iqbal M, Okada S. Suppressive effects of dietary curcumin on the increased activity of renal ornithine decarboxylase in mice treated with a renal carcinoma, ferric nitriltriacetaete. Biochim Biophys Acta 2005; 1740: 357–366.
32 Okazaki Y, Iqbal M, Kawakami N, Yamamoto Y, Toyokuni S, Okada S. A beverage containing fermented black soybean ameliorates ferric nitriltriacetaete-induced renal oxidative damage in rats. J Clin Biochem Nutr 2010; 47: 198–207.
33 Okada S. Prevention of free-radical mediated tissue damage and carcinogenesis induced by low-molecular-weight iron. Biomolals 2003; 16: 99–101.
34 Awai M, Narasaki M, Yamanoi Y, Seno S. Induction of diabetes in animals by parenteral administration of ferric nitriltriacetaete. A model of experimental hemochromatosis. Am J Pathol 1979; 95: 663–673.
35 Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ. Low activities of glutathione peroxidase and glutathione reductase enzymes as factors in the genesis of urinary bladder cancer. Cancer Res 1984; 44: 5086–5091.
36 Carlberg I, Mennervik B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. J Biol Chem 1975; 250: 5475–5480.
37 Tanaka T, Nishiyama Y, Okada K, et al. Induction and nuclear translocation of thoredoxin by oxidative damage in the mouse kidney: independence of tubular necrosis and sulfhydryl depletion. Lab Invest 1997; 77: 145–155.
38 Burrow H, Kanwar RK, Kanwar JR. Antioxidant enzyme activities of iron-saturated bovine lactoferrin (Fe-bLf) in human gut epithelial cells under oxidative stress. Med Chem 2011; 7: 224–230.
39 Yamauchi K, Toida T, Nishimura S, et al. 13-Week oral repeated administration toxicity study of bovine lactoferrin in rats. Food Chem Toxicol 2000; 38: 503–512.
40 Wakabayashi H, Kuwata H, Yamauchi K, Teraguchi S, Tamura Y. No detectable transfer of dietary lactoferrin or its functional fragments to portal
blood in healthy adult rats. *Biosci Biotechnol Biochem* 2004; 68: 853–860.

41 Gosriwatana I, Loreal O, Lu S, Brisson P, Porter J, Hider RC. Quantification of non-transferrin-bound iron in the presence of unsaturated transferrin. *Anal Biochem* 1999; 273: 212–220.

42 Sasaki K, Ikuta K, Tanaka H, et al. Improved quantification for non-transferrin-bound iron measurement using high-performance liquid chromatography by reducing iron contamination. *Mol Med Report* 2011; 4: 913–918.

43 Igarashi M, Watanabe M, Yoshida M, et al. Enhancement of lung carcinogenesis initiated with 4-(N-hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone by Ogg1 gene deficiency in female, but not male, mice. *J Toxicol Sci* 2009; 34: 163–174.

44 Matsuda Y, Saoo K, Hosokawa K, et al. Post-initiation chemopreventive effects of dietary bovine lactoferrin on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in female A/J mice. *Cancer Lett* 2007; 246: 41–46.

45 Li L, Huang L, Vergis AL, et al. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *J Clin Invest* 2010; 120: 331–342.

46 Deguchi J, Kawabata T, Kondo A, Okada S. Transforming growth factor-alpha expression of renal proximal tubules in Wistar rats treated with ferric and aluminum nitrilotriacetate. *Jpn J Cancer Res* 1993; 84: 649–655.

47 Hida AI, Kawabata T, Minamiyama Y, Mizote A, Okada S. Saccharated colloidal iron enhances lipopolysaccharide-induced nitric oxide production in vivo. *Free Radic Biol Med* 2003; 34: 1426–1434.