**Abstract.** Background/Aim: Metabolic syndrome-induced lifestyle-related diseases include diabetes mellitus (DM) and hypertension, and Zn-based compounds have effects on DM. We aimed to investigate the ameliorating effects of bis(hinokitiolato)Zn, \([\text{Zn(hkt)}_2]\) on lipid metabolism in the liver and kidney, histopathologically. Materials and Methods: We used a high-fat diet (HFD)-fed C57BL/6J mouse model and administered a diet containing 10–20 mg Zn/kg body weight (BW) or 20 mg pioglitazone/kg BW as the positive control. After the treatments, we collected blood, liver, and kidney samples and morphologically evaluated the mouse organs for fat accumulation. Results: After a 4-month HFD administration, ectopic fat deposition was detected in the liver and kidney. Furthermore, Zn accumulation in the liver and kidney increased following \([\text{Zn(hkt)}_2]\) treatment, that reduced lipid accumulations and lipid toxicity in these tissues. Conclusion: The results of this study suggest that \([\text{Zn(hkt)}_2]\) could be a novel anti-dyslipidaemia compound for treating diet-induced obesity.

The westernization of eating habits has increased the intake of fat. Metabolic syndrome, which is caused by these changing dietary habits, induces diabetes mellitus (DM) or hypertension (1, 2). There are numerous reports on the effects of a high-fat diet (HFD) intake on health, and the most serious and important are dyslipidaemia, which is characterised by hypertriglyceridaemia, hypercholesterolaemia, and fatty liver (3, 4).

Numerous researchers have reported the effects of many organic or inorganic compounds on various organs, leading to disorders such as fatty liver or diabetic nephropathy (5, 6). Furthermore, other studies have reported that some compounds, even known as beneficial food materials, can worsen a disease condition (7, 8). These reports suggest that careful considerations are required to understand the correct use of these food materials and evaluate their effects on both biochemical parameters and organ morphology. In particular, dyslipidaemia, which is a risk factor for cardiovascular disease, should be prevented.

Pioglitazone is known to exhibit efficacy in the treatment of DM owing to its blood glucose lowering, glycated haemoglobin (HbA1c) improvement, and glucose tolerance ameliorating effects (9, 10). Furthermore, numerous researchers have reported the anti-DM effect of Zn in in vivo experiments (11-13). For example, Zn compound 1) increased Akt-phosphorylation, as well as inhibited tyrosine-protein phosphatase non-receptor type 1B (PTP1B) and phosphatase and tensin homolog (PTEN) in 3T3-L1 adipocytes; and 2) inhibited alpha-glucosidase and 3) activated phosphodiesterase (14-18). Furthermore, only a few studies such as that of Moroki et al. (19) have reported experiments showing the effects of Zn complexes on lipid metabolism. Thus, the effects of Zn compounds on lipid metabolism have not been sufficiently examined yet.

The experimental animals were fed an HFD or high-fructose diet to establish the metabolic syndrome or DM model (20, 21). Therefore, this study examined the histopathology of the livers and kidneys of C57BL/6J mice.
elemental analysis results are as follows: (found/calcd); C 59.40/
ionisation [EI(+)] mode at the Analytical Center of KPU. The
resolution mass spectra were recorded in the positive electron
Center of Kyoto Pharmaceutical University (KPU). The low-
spectrometry (MS, JEOL JMS-SX 102AQQ, JEOL Ltd., Tokyo,
pellet, SHIMADZU Co., Kyoto, Japan), and low-resolution mass
Japan). The elemental analyses were performed at the Analytical
analysis, infrared (IR) absorption (Shimadzu FT-IR 8100A on KBr
CLEA Japan, Inc., (Tokyo, Japan) and were maintained on a 12-h
cumulation. Furthermore, we used pioglitazone treatment as a positive control.

Materials and Methods

Chemicals. Pioglitazone was obtained from Tokyo Chemical Ind.,
Co., Ltd. (Tokyo, Japan). Hinokitiol and all other chemical reagents
were purchased from Wako Pure Chemical Ind., Ltd., (Osaka,
Japan).

Preparation and characterisation of [Zn(hkt)]₂. The [Zn(hkt)₂]
complex was prepared in deionised water/ethanol by mixing
Zn(CH₃COO)₂ and hinokitiol at a 1:2 molar ratio and the solution
was stirred for 12 h at 25°C room temperature. The resulting pale-
yellow precipitate was collected by vacuum filtration, washed
several times with pure ethanol, and dried overnight in vacuo (22).
These prepared complexes were characterised using elemental
analysis, infrared (IR) absorption (Shimadzu FT-IR 8100A on KBr
pellet, SHIMADZU Co., Kyoto, Japan), and low-resolution mass
spectrometry (MS, JEOL JMS-SX 102AQ, JEOL Ltd., Tokyo,
Japan). The elemental analyses were performed at the Analytical
Center of Kyoto Pharmaceutical University (KPU). The low-
solution mass spectra were recorded in the positive electron
ionisation [EI(+)] mode at the Analytical Center of KPU. The
elemental analysis results are as follows: (found/calcd); C 59.40/
59.14%, H 5.91/5.86%. IR spectra (complex/ligand): νC=O=1591
cm⁻¹/1609 cm⁻¹. El(+) MS m/z; 390 [M]+.

Animals. Male C57BL/6J mice (4-week-old) were purchased from
CLEA Japan, Inc., (Tokyo, Japan) and were maintained on a 12-h
light/dark cycle in our central animal facility. They were housed in
an air-conditioned room at a temperature and humidity of 23±1°C
and 60±10%, respectively. All animals were allowed free access to
the standard food (Table I) and tap water. All animal experimental
protocols were approved by the Experimental Animal Research
Committee of KPU and were performed according to the Guidelines
for Animal Experimentation of KPU.

Dietary formulations with pioglitazone and [Zn(hkt)₂]. All animals
had free access to water and semi-synthetic HFDs that were high in
sugar and, therefore, hypercaloric (composition of the basal diet
[%]: sucrose, 30%; lard fat, 18.2%; and casein, 18.2%; Kobe
Women’s University special diets, Kobe, Japan). The diets were
prepared for all groups using AIN-93N and a mixture of a standard
diet (Oriental East Co., Ltd., Tokyo, Japan) as shown in Table I. The
dose of pioglitazone was determined based on that used in previous
studies (23-25), and we prepared an HFD containing 20 mg/kg BW
pioglitazone for the drug-treated group. Previous reports showed
that the effective dose of Zn complexes for anti-DM activity is
approximately 15 mg Zn/kg BW. Furthermore, we prepared an HFD

| CNT | Pioglitazone | [Zn(hkt)₂] |
|-----|-------------|-----------|
|     |             | 10 mg Zn  | 15 mg Zn  | 20 mg Zn  |
| Casein | 18.2       | 18.2      | 18.2      | 18.2      |
| Sucrose | 30.0      | 30.0      | 30.0      | 30.0      |
| Lard  | 18.2       | 18.2      | 18.2      | 18.2      |
| Vitamin mix. AIN 93N | 0.9       | 0.9       | 0.9       | 0.9       |
| Mineral mix. AIN 93N | 3.2       | 3.2       | 3.2       | 3.2       |
| Cellulose | 4.5      | 4.5       | 4.5       | 4.5       |
| L-Cystine | 0.2       | 0.2       | 0.2       | 0.2       |
| Choline bitartrate | 0.2       | 0.2       | 0.2       | 0.2       |
| t-Butylhydroquinone | 0.0007  | 0.0007    | 0.0007    | 0.0007    |
| Cornstarch | 15.5    | 15.5      | 15.5      | 15.5      |
| Sample | 0.00       | 0.02      | 0.04      | 0.08      |
| Water | 9.1        | 9.1       | 9.1       | 9.1       |
| Total (%) | 100      | 100       | 100       | 100       |

CNT: Control; [Zn(hkt)₂]: bis(hinokitiolato)Zn complex.

| Sample | Body weight (g) | BGL (mg/dL) |
|--------|-----------------|-------------|
|        | Before          | After       | Before      | After      |
| C57BL/6J |               |             |             |            |
| CNT    | 20.1±0.9       | 37.5±3.4    | 101±33      | 160±27     |
| Pioglitazone | 19.0±1.2 | 38.3±3.4    | 101±43      | 142±23     |
| [Zn(hkt)₂] | 19.2±0.8 | 30.0±4.4**  | 146±31      | 125±6*     |

*p<0.05 and **p<0.01 vs. control (CNT) group. [Zn(hkt)₂]:
bis(hinokitiolato)Zn complex.
containing 10-20 mg/kg BW [Zn(hkt)2] for the [Zn(hkt)2]-treated group (26, 27).

Animal experiments. All animals used for the in vivo study were 6-weeks-old. We purchased 20 C57BL/6J mice, which were adapted to the environment. Then, the mice were divided into three groups fed either a basal HFD (CNT) or a basal HFD containing pioglitazone or supplemented with [Zn(hkt)2]. The C57BL/6J mice were allowed free access to a solid HFD for the CNT group, and an HFD with pioglitazone or [Zn(hkt)2] for the drug-treated groups, and tap water for 4 months. Blood glucose levels (BGL) were measured once weekly, and the BW, food intake, and water consumption were monitored every 3 days. Blood samples for the analysis of BGL were obtained from the tail vein of the normal C57BL/6J mice and measured using the glucose oxidase method (Glucocard, Arkray, Kyoto, Japan). In the histopathological experiments, we randomly chose three mice from each group.

Tissue fixation and processing. At 4 months of age, the liver and kidney tissues from the C57BL/6J mice were removed, fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3-μm thickness, and stained with haematoxylin and eosin (H&E). The weights of the liver and kidneys were also measured.

Morphometric analysis of liver and kidney. H&E-stained sections of each tissue were scanned using a high-resolution digital slide scanner (NanoZoomer 2.2 Digital Pathology, Hamamatsu Photonics, Hamamatsu, Japan) to prepare digital images. The ndpi image files were opened in the colour mode using the NDP.view2 software (Hamamatsu, Japan) to prepare digital images. The ndpi image files were used for the quantitation assay for determining the Zn concentrations using ICP-MS.

Blood biochemical analysis. At euthanasia, blood samples were withdrawn from the abdominal inferior vena cava of mice exsanguinated under anaesthesia with ether, and centrifuged for 10 min at 650 x g at 4°C. The obtained serum samples were analysed to detect the concentration of blood urea nitrogen (BUN), glutamic pyruvic transaminase/alanine aminotransferase (GPT/ALT), glutamic oxaloacetic transaminase/aspartate aminotransferase (GOT/ALT), glutamic pyruvic transaminase/alanine aminotransferase (GPT/ALT), glutamic oxaloacetic transaminase/aspartate aminotransferase (GOT/ALT), triglyceride (TG), total cholesterol (TCHO), and high-density lipoprotein (HDL) using Fuji DRI-CHEM 4000sV (FUJIFILM Medical Co. Ltd., Tokyo, Japan). GPT/ALT and GOT/AST levels reflect hepatocyte damage and, therefore, they are valuable biomarkers of chemical-induced hepatotoxicity. TG and TCHO indicate the rate of cholesterol metabolism and TG, TCHO, and HDL were used as valuable biomarkers for lipid metabolism.

Zn concentrations in liver and kidney. Liver and kidney samples were collected from all three groups, and the Zn levels were determined using inductively coupled plasma (ICP)-MS (Agilent 7700x/Mass Hunter; Agilent Technologies, Inc., Santa Clara, CA, USA). To determine the total amount of Zn in the liver and kidney, the collected samples were washed with saline and approximately 30 mg of vacuum-dried tissue was obtained, heated to 180°C, and 2 mL each of 60% nitric acid (HNO3), 60% perchloric acid (HClO4), and 30% hydrogen peroxide (H2O2) was added. This procedure was repeated until all the organic material was removed. After cooling the samples to room temperature, the residues were re-suspended in 9 mL 5% HNO3. These solutions were used for the quantitation assay for determining the Zn concentrations using ICP-MS.

Statistical analysis. All discrete values except for the histopathological results were expressed as the means±standard deviation (SD) and analysed using a one-way analysis of variance (ANOVA) and Dunnet’s multiple comparison tests. Differences were considered significant at p<0.05 or p<0.01.

Results

General remarks. There were no significant differences in the BW on day 1 between the groups. At 4 months after sample treatment, the BW of the [Zn(hkt)2] group significantly decreased compared with that of the CNT group (p<0.01).

Table III. Serum parameters of control (CNT), pioglitazone-, and bithinokitiolatoZn complex ([Zn(hkt)2])-treated C57BL/6J mice.

|                | TG     | TCHO   | HDL    | BUN    | GPT/ALT | GOT/AST | Liver weight | Kidney weight |
|----------------|--------|--------|--------|--------|---------|---------|--------------|---------------|
|                | (mg/dL)| (U/L)  | (g of wet weight) |
| C57BL/6J       |        |        |        |        |         |         |              |               |
| CNT            | 108±20 | 135±35 | 114±5  | 21.2±1.7| 16±5   | 43±5   | 1.2±0.1      | 0.37±0.05     |
| Pioglitazone   | 108±13 | 141±12 | 102±6* | 17.1±0.1**| 43±17**| 55±8** | 1.3±0.2      | 0.30±0.02     |
| [Zn(hkt)2]     | 75±19**| 122±25 | 96±10**| 17.7±2.4**| 14±6   | 41±6   | 0.9±0.1      | 0.30±0.03     |

*p<0.05 and **p<0.01 vs. CNT (control); TG: triglyceride; TCHO: total cholesterol; HDL: high-density lipoprotein; BUN: blood urea nitrogen; GPT/ALT: glutamic pyruvic transaminase/alanine aminotransferase; GOT/ALT: glutamic oxaloacetic transaminase/aspartate aminotransferase.
Blood biochemical analysis. The [Zn(hkt)₂]_treated group showed a significantly decreased BGL compared to the CNT group at the end of the treatment period (Table II). The BUN levels of the pioglitazone- and [Zn(hkt)₂]-treated groups significantly decreased (p<0.01). Furthermore, the GPT/ALT and GOT/AST levels in the pioglitazone-treated group increased significantly (p<0.01, Table III).

Liver morphology. After administering pioglitazone or [Zn(hkt)₂] for 4 months, the mouse livers and kidneys were collected and weighed. The weights of both the livers and kidneys of the mice in the various groups were not significantly different (Table III).

The histopathological changes induced by 4-month ingestion of HFD or the HFD containing pioglitazone or [Zn(hkt)₂] are summarised in Table IV, and the histopathological observations are shown in Figure 1. The livers of the CNT and the pioglitazone-treated group showed hepatocellular fatty changes after a 4-month ingestion of HFD. Fatty changes were mainly observed in the centrilobular area in the CNT and the pioglitazone-treated groups. However, although the [Zn(hkt)₂]-treated group consumed the HFD, they showed a reduction in the level of fatty change.

Kidney morphology. Fatty changes were observed in the proximal tubular epithelia of the CNT group while [Zn(hkt)₂] treatment reduced the tubular fatty changes (Table IV). The histopathological observations are shown in Figure 2.

Organ distribution of Zn. The Zn accumulation in the livers and kidneys of the C57BL/6J mice was determined after the treatment period using ICP-MS. The pioglitazone group showed slight decreases in both the hepatic and renal Zn concentration compared with the CNT group. In contrast, the hepatic and renal Zn concentrations of the [Zn(hkt)₂] group increased compared with those of the CNT group (Table V).
results suggest that the untreated C57BL/6J mice developed hypertriglyceridaemia and hypercholesterolaemia as well as fatty liver disease. These blood biochemical parameters were consistent with the fat deposition in the liver. Furthermore, when the mice were fed the [Zn(hkt)₂], the Zn concentration of the liver increased compared with the untreated mice. These data suggest that Zn activated lipid metabolism such as beta-oxidation by activating or inhibiting some enzymes. It is well known that lipid accumulation in the liver is inhibited when 1) inflammation is reduced or 2) lipid absorption from food is regulated. Adiponectin is an adipocytokine with anti-inflammatory effects. Moreover, adiponectin activates beta-oxidation by activating AMP-activated protein kinase (AMPK). Yamane et al. (22) reported that [Zn(hkt)₂] induced the expression levels of adiponectin in KK-A₁ mice. Zn also has anti-inflammatory effects throughout the body and, thus, in this study, the [Zn(hkt)₂] group showed less lipid accumulation in the liver.

Diabetic nephropathy often occurs during the progression of type 2 diabetes. Diabetic nephropathy is pathologically characterised by glomerular hypertrophy and hyperfiltration, thickening of the basement membrane, and accumulation of the extracellular matrix (31, 32). Numerous studies have reported that HFD induced metabolic syndrome or DM. Renal alterations such as lipid accumulation lead to increased organ weight and inflammatory infiltrates. The increased release of proinflammatory cytokines may be associated with kidney damage and oxidative stress (33-35). Moreover, numerous studies of renal function in DM and diabetic nephropathy evaluated the size of the glomerulus to determine the glomerular hypertrophy or collagen IV deposition as a measure of matrix deposition (36).
Thus, in this study, we evaluated renal function by observing H&E-stained histological sections and found that the CNT group showed increased lipid deposition while both the pioglitazone- and [Zn(hkt)2]-treated groups showed a reduction. Hirasawa et al. (37) reported that pioglitazone inhibited the mRNA expression of the receptor for advanced glycation end-products (RAGE) and transforming growth factor (TGF)-β. Thus, they concluded that pioglitazone improves obesity type diabetic nephropathy (37). The results of our study suggest that [Zn(hkt)2] may have direct effects on the kidney similar to pioglitazone.

In conclusion, we evaluated the ectopic fat accumulation induced by an HFD using normal C57BL/6J mice. We found that pioglitazone and [Zn(hkt)2] treatments both reduced the liver and kidney lipid accumulation, which ameliorated the lipid toxicity. [Zn(hkt)2] has the potential to protect hepatic and renal function from the lipid toxicity, and its effects are likely higher than those of pioglitazone. This study suggests that [Zn(hkt)2] could be a novel compound for the treatment of dyslipidaemia.

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