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Syntheses of Cu(II), Ni(II), and Zn(II) complexes with 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone and evaluation of their antidiabetic effects

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Abstract

The increasing number of patients with diabetes has recently become a serious problem following changes in diet and lifestyle. Various drugs are used to treat diabetes; however, there are serious concerns regarding their physical and mental side effects. Zinc, copper, and nickel are trace elements present in the body that are known to have insulin-like effects although nickel is not essential to mammals. Here, we focused on metal complexes with the 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone (2-APTC) ligand, which has a six-coordinate octahedral structure with an S_2N_4-type coordination mode. For single oral administration experiments, [Zn(2-APTC)$_2$], zinc sulfate (10 mg Zn/kg) and [Cu(2-APTC)$_2$], and copper sulfate (3 mg Cu/kg) were orally administered to 5-week-old ddy mice fasted for 12 h. Moreover, of [Zn(2-APTC)$_2$] was administered daily to KK-A^y mice, a type 2 diabetes model. In a single oral administration experiment, [Zn(2-APTC)$_2$] showed a significant increase in plasma zinc concentration compared to zinc sulfate. Moreover, the 28-d administration of [Zn(2-APTC)$_2$] resulted in a significant decrease in the blood glucose level. This suggests that [Zn(2-APTC)$_2$] has a higher absorption of the complexes than [Cu(2-APTC)$_2$] after oral administration and is expected to have more antidiabetic activity. However, blood urea nitrogen, aspartate aminotransferase, and alanine transaminase levels increased, suggesting that [Zn(2-APTC)$_2$] administration affects liver and kidney functions. Moreover, hematoxylin and eosin staining showed that [Zn(2-APTC)$_2$] ameliorated fatty liver and exerted antidiabetic effects. Here, we report for the first time that a zinc complex with a six-coordinated octahedral structure, with 2-APTC as a ligand, exhibits antidiabetic effects.

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Introduction

According to the International Diabetes Federation, the number of adults with diabetes worldwide was 367 million in 2014, and this figure is expected to reach 591.9 million by 2035. In Japan, the number of people with diabetes is estimated to be approximately 7.2 million, which is the 10th largest population worldwide [1]. Diabetes mellitus is a chronic hyperglycemic disorder characterized by impaired glucose tolerance and high glucose levels caused by impaired glucose, lipid, and protein metabolism due to an absolute or relative lack of insulin. Continuous poor glycemic control can lead to conditions such as atherosclerosis and three major complications: retinopathy, nephropathy, and neuropathy. Diabetes is classified into two types: type 1, which is caused by autoimmune diseases against beta islet cells, and type 2, which is caused by lifestyle factors such as heredity, obesity, and stress. Ninety percent of patients with diabetes have type 2 diabetes. The basic treatment for type 2 diabetes is changing diet and exercise habits if these do not improve the condition, drug therapy is introduced. Various drugs are currently used to treat diabetes mellitus [2, 3]. However, there are concerns about the pain associated with the self-injection of insulin and the possibility of life-threatening side effects such as hypoglycemia and coma caused by hypoglycemic agents [4]. In recent years, the number of patients with type 2 diabetes is expected to increase not only in developed countries, where people tend to overindulge and become over-nourished, but also in developing countries, where lifestyles have become more affluent owing to recent economic growth.

Zinc is an essential trace element; approximately 2 g of zinc is distributed in the human body. It is a crucial substance involved in various processes in the body, such as promoting growth and metabolism, normalizing the sense of taste and smell, normalizing appetite, maintaining brain function, inhibiting cell aging and cancer, and maintaining immunity [5]. In 1980, Coulston et al. first reported that zinc has insulin-like effects on rat adipocytes *in vitro* [6]. In 1992, Chen et al. reported that drinking water containing a high concentration of zinc chloride for eight weeks can lower the fasting blood glucose levels of ob/ob mice, a model of type 2 diabetes [7]. In 2000, a zinc complex with insulin-like activity was first synthesized to enhance the insulin-like effect of zinc. To improve the quality of life of patients with diabetes, various metal complexes, including zinc, have been synthesized and evaluated for their antidiabetic effects as candidate compounds for new, alternative antidiabetic drugs to conventional synthetic hypoglycemic agents with side effects and insulin injections that impose a serious mental and physical burden [8-11]. Copper is also known as an essential trace element, similar to zinc, whereas nickel is not an essential element, but is present in the body of animals. Complexes containing these metals reportedly exhibit antidiabetic effects [12,13].

Thiosemicarbazone compounds contain structures shown in **Fig. 1** and are urea derivatives synthesized from urea and hydrazine. Thiosemicarbazones have been widely studied since the discovery of the antibacterial activity of acetone thiosemicarbazone in the late 1940s. Thiosemicarbazone compounds have many pharmacological effects and remain widely studied. In some cases, they are used as medical products and raw materials for agricultural chemicals [14-16]. In this study, we focused on metal complexes with 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone (2-APTC), one such thiosemicarbazone compound, as a ligand. Many metal complexes with thiosemicarbazone derivatives have been reported; however, most have been evaluated for their antibacterial, anticancer, and antiviral activities [17-21]. If metal complexes with 2-APTC ligands are found to have antidiabetic effects, such metal complexes with thiosemicarbazone compounds could be new candidates for antidiabetic drugs in the future. Metal complexes with 2-APTC as a ligand have a six-coordinated octahedral structure with the S_2N_4 coordination mode. As complexes with the same six-coordinate octahedral structure, di(ethylenediamine pentaacetic acid) zinc(II) and ethylenediamine-*N*, *N*, *N*′, *N*′-tetraacetic acid zinc(II) complexes were synthesized and evaluated, but none of them showed insulin-like activity (unpublished data). Therefore, in our previous study, we examined whether the complex with 2-APTC as a ligand is the first zinc complex with a six-coordinate octahedral structure that exhibits antidiabetic activity.

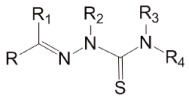


Fig. 1 The drawing represents the general formula for thiosemicarbazones. R_1, R_2, R_3, R_4 = H, or any organic substituent

Materials and methods

Chemicals

Phenyl isothiocyanate and 2-acetylpyrazine were purchased from Tokyo Chemical Industry Co., Ltd. (Osaka, Japan). Hydrazine hydrate, collagenase (Type II), (±)-epinephrine, and bovine serum albumin (BSA; fraction V) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Zinc perchlorate hexahydrate ($Zn(ClO_4)_2{\cdot}6H_2O$), nickel perchlorate hexahydrate ($Ni(ClO_4)_2{\cdot}6H_2O$), sodium acetate (CH_3COONa), zinc (II) sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$), copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$), nickel (II) sulfate hexahydrate ($NiSO_4{\cdot}6H_2O$), NEFA-C Test Wako, glucose, sodium chloride (NaCl), calcium chloride ($CaCl_2$), magnesium sulfate ($MgSO_4$) potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), zinc and copper standard solution (for atomic absorption spectrophotometry), nitric acid (61%, for toxic metal determination), perchloric acid (60%, for toxic metal determination), polyethylene glycol 400 (PEG400), gum arabic(acasia), and formalin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Copper perchlorate hexahydrate ($Cu(ClO_4)_2{\cdot}6H_2O$) was purchased from KANTO CHEMICAL CO., INC. (Kyoto, Japan). Acetic acid (CH_3COOH), sodium dihydrogen phosphate and disodium hydrogen phosphate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Dimethyl sulfoxide (DMSO), and hydrogen peroxide (30% for atomic absorption spectrophotometry) were purchased from KISHIDA CHEMICAL Co.,LTD. (Osaka, Japan). The solid feed (MF) was purchased from Oriental Yeast Co.,Ltd., Osaka, Japan. The mouse leptin immunoassay kit was purchased from R&D Systems Corporation. Fuji Dry Chem slides for blood urea nitrogen (BUN), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were purchased from FUJIFILM Corporation (Tokyo, Japan), and an ultra-sensitive mouse insulin assay kit was purchased from Morinaga Institute of Biological Science, Inc. (Yokohama, Japan). All other organic solvents and inorganic reagents were of first grade or special grade. Ultrapure water was obtained from a Milli-Q Ultrapure Water System (Millipore, Japan). All aqueous solutions were prepared using ultrapure water.

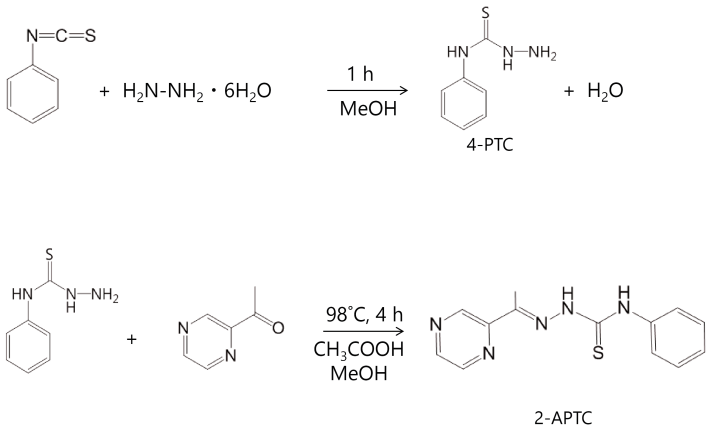
Analytical instruments

The synthesized complexes were characterized using elemental analysis, infrared absorption spectroscopy (IR), and low-resolution mass spectrometry (EI, FAB-MS) to evaluate their physical properties. IR was performed by the potassium bromide tablet method using a Shimadzu FTIR-8100A (SHIMADZU CORPORATION, Kyoto, Japan), and molar absorption coefficients were measured using an Agilent-8453 spectrometer (Agilent Technologies Japan, Ltd., Tokyo, Japan). Elemental analysis and mass spectrometry were performed by staff at the Kyoto Pharmaceutical University (KPU) Collaborative Research and Application Center.

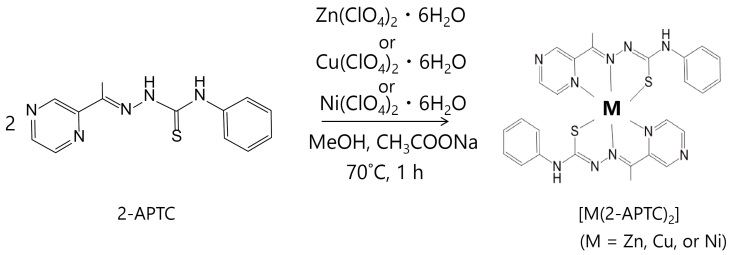
Synthesis method

Synthesis of 2-APTC (Scheme 1)

Phenyl isothiocyanate (10 mmol) and hydrazine hydrate (10 mmol) in methanol (MeOH) were stirred for 2 h at 22-25 °C. After stirring, the white precipitate produced was filtered off by filtration, and the filtrate was evaporated at 45 °C. Precipitated white crystals (*N*(4)-of (4-PTC)) was collected on a glass filter by filtration and dried in a vacuum desiccator. 2-acetylpyrazine



Scheme 1 | Synthesis of 2-APTC



Scheme 2 | Synthesis of metal complexes with 2-APTC as a ligand

(3 mmol) and CH_3COOH, a catalyst, were added to 4-PTC (3 mmol) in MeOH and refluxed at 98 °C for 4 h. After refluxing, the product was cooled overnight at room temperature, and the precipitated yellow crystals (2-APTC) were collected on a glass filter by suction filtration and dried in a vacuum desiccator.

Synthesis of [Zn(2-APTC)$_2$] (Scheme 2)

Each of CH_3COONa (0.4 mmol), 2-APTC (0.4 mmol), and $Zn(ClO_4)_2{\cdot}6H_2O$ (0.2 mmol) was dissolved in MeOH. The $Zn(ClO_4)_2{\cdot}6H_2O$ solution was stirred and the 2-ATPC solution with CH_3COONa was added by drop mixing. The mixed solution was stirred for a 1 h at 70 °C using a mantle heater. After stirring, the resulting yellow precipitate was collected on a glass filter via suction filtration and dried in a vacuum desiccator.

Synthesis of [Cu(2-APTC)$_2$] (Scheme 2)

CH_3COONa (0.4 mmol), 2-APTC (0.4 mmol) and $Cu(ClO_4)_2{\cdot}6H_2O$ (0.2 mmol) were dissolved in MeOH, respectively. The $Cu(ClO_4)_2{\cdot}6H_2O$ solution was stirred and the 2-ATPC solution with CH_3COONa was added by drop mixing. The mixed solution was stirred for a 1 h at 70 °C using a mantle heater. After stirring, the resulting dark green precipitate was collected on a glass filter via suction filtration and dried in a vacuum desiccator.

Synthesis of [Ni(2-APTC)$_2$] (Scheme 2)

CH_3COONa (0.4 mmol), 2-APTC (0.4 mmol) and $Ni(ClO_4)_2{\cdot}6H_2O$ (0.2 mmol) were each dissolved in MeOH. The $Ni(ClO_4)_2{\cdot}6H_2O$ solution was stirred and the 2-ATPC solution with CH_3COONa was added by drop mixing. The mixed solution was stirred for a 1 h at 70 °C using a mantle heater. After stirring, the resulting dark reddish-brown precipitate was collected on a glass filter via suction filtration and dried in a vacuum desiccator.

Animals

Male Wistar rats (7–8 weeks old) were purchased from SHIMIZU Laboratory Supplies Co., Ltd. (Kyoto, Japan). The animals were kept in plastic breeding cages with *ad libitum* access to MF and tap water under a 12-h photoperiod at the KPU Central Animal Research Center until the start of the experiment.

Five-week-old ddy mice were purchased from SHIMIZU Laboratory Supplies Co., Ltd. The animals were kept in plastic breeding cages with *ad libitum* access to MF and breeding water of the facility under a 12-h photoperiod at the Bioscience Research Center until the start of the experiment. Five-week-old KK-A^y mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). During the four-week feeding, blood glucose levels and body weights were measured once a week to ensure that hyperglycemia was sustained before the experiment was conducted. KK-A^y mice were housed individually in cages. All animal experiments were approved by the KPU experimental committee and were conducted in accordance with the KPU guidelines for animal experiments (Animal experiment approval no. 17-13-013).

Evaluation of *in vitro* insulin-like activity (free fatty acid release inhibition test) [22]

Seven- or eight-week-old male Wistar rats were killed by exsanguination under ether anesthesia, and then the white adipose tissue around the epididymis was removed and incubated in Krebs-Ringer Bicarbonate buffer containing collagenase (120 mM NaCl, 1.27 mM $CaCl_2$, 1.20 mM $MgSO_4$, 4.75 mM KCl, 1.20 mM KH_2PO_4, 24 mM $NaHCO_3$, 2% BSA, pH 7.4) (KRB-BSA), adipocytes were subdivided by incubating for 1 h at 37 °C. The subdivided adipocytes were filtered through gauze and washed thrice with KRB-BSA. The cells were then dispensed so that the concentration of cells was 1.5×10^6 cells/240 μL KRB-BSA. Next, glucose was added to all the cell solutions to a final concentration of 5 mM, and then the samples were added and preincubated for 30 min at 37 °C. The synthesized complexes, ligands, and metal ions were used as test samples. $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, and $NiSO_4{\cdot}6H_2O$ were used as the metal ion indicators in particular. The metal ions were dissolved in ultrapure water, whereas the other samples, complexes, and ligands were dissolved in DMSO because they were insoluble in water. The final concentration of DMSO in the reaction mixture was 2%. Finally, epinephrine was added to make the final concentration 10 μM and the solutions were incubated for 3 h at 37 °C. Then, the cells were centrifuged at 825 g, 4°C for 10 min and the concentration of free fatty acid (FFA) in KRB-BSA was determined by NEFA-C Test Wako Kit. Insulin-like activity was evaluated as the 50% inhibitory concentration (IC_{50}), which is the concentration of the complex that inhibits 50% of the amount of FFA released from adipocytes by epinephrine stimulation as 100%.

Evaluation of intestinal absorption properties of complexes

Five-week-old male ddy mice were fasted for 12 h and then orally administered [$Cu(2\text{-}APTC)_2$] and [$Zn(2\text{-}APTC)_2$] dissolved in PEG400 at 3 mg Cu/kg body weight (BW) and 10 mg Zn/kg BW, respectively, in a single dose. Blood samples were collected from the abdominal vena cava under isoflurane anesthesia 1, 2, 4, 6, and 8 h after oral administration. At this time, a single oral administration of $CuSO_4{\cdot}5H_2O$ and $ZnSO_4{\cdot}7H_2O$, dissolved in 5% acacia solution respectively, were also administered to compare the absorption rate of complex state and ionic state. The collected blood samples were centrifuged at 825 g, 4 °C for 10 min, twice. The supernatant was collected as a plasma sample. These plasma samples were weighed into 50 mL tall beakers and wet ashing with 5 mL of 60% nitric acid, 5 mL of 60% perchloric acid, and 5 mL of 30% hydrogen peroxide on a 140 °C hot plate. This procedure was repeated until all organic materials were removed. After cooling to room temperature, the samples were resuspended in 1% HNO_3. The plasma concentrations of Cu and Zn were determined using an Atomic Absorption Spectrophotometer (AA-6300, Shimadzu Rika Co., Ltd., Kyoto).

The evaluation of *in vivo* antidiabetic effects of [$Zn(2\text{-}APTC)_2$]

Nine-week-old KK-A^y mice were divided into the control (Cont. group, n=7) and treatment groups ([$Zn(2\text{-}APTC)_2$] group, n=7). The Cont. group was orally administered PEG 400 and the [$Zn(2\text{-}APTC)_2$] group was orally administered 15 mg Zn/kg BW of [$Zn(2\text{-}APTC)_2$] dissolved in PEG 400 continuously for 28 d. Body weight, blood glucose levels, food intake, and water intake were measured daily during the administration period. Blood glucose levels were measured using a Glucocard system (ARKRAY, Inc., Kyoto, Japan). After treatment with [$Zn(2\text{-}APTC)_2$] for 28 d, the mice were fasted for 12 h, and an oral glucose tolerance test (OGTT) was performed. Mice were orally administered a 1 g/kg BW glucose solution, and blood glucose levels were measured at 0, 15, 30, 45, 60, 90, and 120 min by collecting blood samples from the tail vein. HbA1c levels were measured in blood collected from the tail vein using an immunoassay with a DCA2000 analyzer (Bayer-Sankyo, Osaka, Japan) and a DCA2000 HbA1c cartridge (Siemens, Erlanger, Germany).

After the OGTT and HbA1c measurements, the mice were fasted for 12 h, anesthetized with isoflurane, and blood was collected from the abdominal vena cava using a heparinized syringe. The organs were collected after blood collection. Blood samples were centrifuged twice at 4 °C, 825 g for 10 min and the supernatant was collected to serve as the plasma samples. After 48 h, the solution was replaced with 70% ethanol and stored at 4 °C. Plasma and other organs were stored at -80 °C. Plasma levels of BUN, ALT, and AST were measured using a Fuji Dry Chem system (FUJIFILM Corporation), and those of insulin and leptin were determined using a Morinaga Mouse Insulin Kit and a leptin immunoassay kit (R&D Systems, Minneapolis, MN, USA), respectively.

Determinations of Zn and Cu concentrations in organs

To determine Zn and Cu concentrations in plasma and collected organs of treated mice, the samples were heated with 61% HNO_3, 60% $HClO_4$, and 30% H_2O_2 at 180 °C by the wet ashing method. The concentrations of Zn and Cu were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700x/Mass Hunter; Agilent Technologies Inc., Santa Clara, CA, USA).

Tissue fixation and processing

To evaluate tissue staining, a portion of the liver and pancreas of the treated mice was placed in 10% formaldehyde buffer. The pancreas and liver were immersed in 70% ethanol, dehydrated in ethanol and xylene, and embedded in paraffin. The organs were then fixed with paraffin and thinly sliced. After deparaffinization with xylene, 95 and 100% ethanol, and staining and washing with hematoxylin and eosin solutions, the samples were dehydrated with 80, 90, and 100% ethanol. Each glass slide was then covered with a cover glass with sealing agent, overlaid on the tissue, and dried. Each sample was examined under a biological microscope (BA210E, SHIMADZU CORPORATION). For the pancreas, 50% of the islets in each mouse were randomly selected and their area was measured. For the liver, seven different areas of each sample were captured and the percentage of fat in each image was evaluated as follows:0 for 0-10%, +1 for 10-30%, +2 for 30-50%, and +3 for > 50%, which were then averaged and evaluated.

Statistical processing

All experimental results are expressed as mean ± standard deviation (SD), and the *t*-test was used to evaluate significant differences. Only fatty liver was evaluated using the Cochran–Armitage test.

Results

Evaluation of physicochemical properties of 2-APTC

The yield was approximately 10%. Elemental analysis (%) values of the molecular formula ($C_{13}H_{13}N_5S_1$), theoretical values (H:4.83, C:57.54, N:25.81), measured values (H:4.7, C:57.71, N:25.94), IR values:1169 cm^{-1}, 1530 cm^{-1} and 1554 cm^{-1}, EI-MS value:271 m/z.

Evaluation of physicochemical properties of [Zn(2-APTC)$_2$], [Cu(2-APTC)$_2$], and [Ni(2-APTC)$_2$]

The yield of [Zn(2-APTC)$_2$] was about 44%. Elemental analysis (%) values of the estimated complex molecular formula ($C_{26}H_{24}N_{10}S_2Zn$), theoretical values (H:3.99, C:51.52, N:23.11), measured values (H:3.60, C:51.56, N:22.81), IR values: 1167 cm^{-1}, 1508 cm^{-1}, 1601 cm^{-1}, and FAB-MS value: 605 m/z. Considering the C content as a criterion, the purity of the synthesized [Zn(2-APTC)$_2$] was considered to be 100%.

The yield of [Cu(2-APTC)$_2$] was approximately 69%. Elemental analysis (%) values of the estimated complex molecular formula ($C_{26}H_{24}N_{10}S_2Cu{\cdot}0.5H_2O$), theoretical values (H:4.12, C:50.84, N:23.19), measured values (H:3.91, C:50.91, N:22.51), IR values: 1143 cm^{-1}, 1493 cm^{-1}, 1601 cm^{-1}, and FAB-MS value: 603 m/z. Considering the C content as a criterion, the purity of the synthesized [Cu(2-APTC)$_2$] was considered to be 98%.

The yield of [Ni(2-APTC)$_2$] was approximately 71%. Elemental analysis (%) values of the estimated complex molecular formula ($C_{26}H_{24}N_{10}S_2Ni{\cdot}0.7H_2O$), theoretical values (H:4.18, C:51.02, N:22.89), measured values (H:3.84, C:50.73, N:22.95), IR values: 1146 cm^{-1}, 1505 cm^{-1}, 1601 cm^{-1}, and FAB-MS value: 599 m/z. Considering the C content as a criterion, the purity of the synthesized [Ni(2-APTC)$_2$] was considered to be 97%.

Evaluations of *in vitro* insulin-like activity

As shown in **Table 1**, all synthesized complexes of [Zn(2-APTC)$_2$], [Cu(2-APTC)$_2$], and [Ni(2-APTC)$_2$] showed dose-dependent inhibitory effects on free fatty acid release, that is, an insulin-like effect. In particular, the IC_{50} values of [Zn(2-APTC)$_2$] and [Cu(2-APTC)$_2$] were 16.9 ± 6.2 μM and 11.3 ± 3.6 μM, respectively, and their inhibitory effects were seen at much lower concentrations than that of $ZnSO_4{\cdot}7H_2O$. This insulin-like effect was also observed for $NiSO_4{\cdot}6H_2O$ used as an indicator of Ni ion, but the effect was far weaker than those of $ZnSO_4{\cdot}7H_2O$ and $CuSO_4{\cdot}5H_2O$. However, there was no insulin-like effect of ligand 2-APTC.

Table 1 Result of insulin-like effect. Estimated IC_{50} values of each compound for FFA release from rat adipocytes treated with epinephrine.
Data are expressed as the means ± SD for 3 independent experiments. **Significant at $P < 0.01$ vs. $ZnSO_4 \cdot 7H_2O$. ## Significant at $P < 0.01$ vs. $CuSO_4 \cdot 5H_2O$.

Compounds	IC_{50} (μM)
$ZnSO_4 \cdot 7H_2O$	531 ± 52
$CuSO_4 \cdot 5H_2O$	298 ± 67
$NiSO_4 \cdot 6H_2O$	Low activity
[Zn(2-APTC)$_2$]**	16.9 ± 6.2
[Cu(2-APTC)$_2$]##	11.3 ± 3.6
[Ni(2-APTC)$_2$]	405 ± 64
2-APTC	None

Evaluations of Cu(II) and Zn(II) complex for intestinal absorption

The plasma Cu concentration after oral administration of [Cu(2-APTC)$_2$] increased slightly at 4 h, but decreased to almost the same level as that before oral administration at 8 h (**Fig. 2 [A]**). This indicated that the absorption of [Cu(2-APTC)$_2$] from the gastrointestinal tract into the blood circulation was quite low. In addition, there was little difference in the plasma Cu concentrations after a single oral administration of [Cu(2-APTC)$_2$] and ionic $CuSO_4 \cdot 5H_2O$. However, after oral administration of [Zn(2-APTC)$_2$], the plasma concentration of Zn significantly increased until 2 h after administration, and then decreased slowly until 8 h to almost the same level as before administration. It was also found that plasma Zn concentrations after single oral administration of [Zn(2-APTC)$_2$] were considerably higher than those of ionic state $ZnSO_4 \cdot 7H_2O$ (**Fig. 2 [B]**). Although the insulin-like activity of [Zn(2-APTC)$_2$] in the *in vitro* FFA test was lower than that of [Cu(2-APTC)$_2$], [Zn(2-APTC)$_2$] was more readily absorbed than [Cu(2-APTC)$_2$] into blood circulation from the gastrointestinal tract after oral administration.

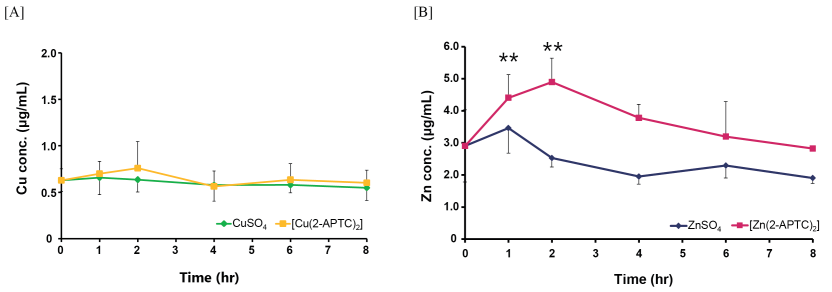


Fig. 2 Changes of Cu [A] and Zn [B] concentration in plasma by single-dose administration. Values are means ± SD for 5 mice. **Significant at P < 0.01 vs. $ZnSO_4$.

Antidiabetic effect of [Zn(2-APTC)$_2$]

Changes in body weight, blood glucose levels, food intake, and water intake were measured during the treatment period, as shown in **Fig. 3**. No significant differences were observed in body weight (**Fig. 3[A]**). The blood glucose levels, [Zn(2-APTC)$_2$] group showed the significant decrease compared with the Cont. group on day 12 and beyond (**Fig. 3 [B]**). The [Zn(2-APTC)$_2$] group had a lower food intake than the Cont. group; however, the [Zn(2-APTC)$_2$] group ingested approximately the same amount from day 23. The [Zn(2-APTC)$_2$] group had significantly lower water intake than the Cont. group. These results showed that the administration of [Zn(2-APTC)$_2$] decreased blood glucose levels and water intake and improved hyperglycemia and polydipsia, which are symptoms of DM. In the OGTT, the increase in blood glucose levels in the [Zn(2-APTC)$_2$] group was significantly suppressed compared with that in the Cont. group. The AUC was also significantly decreased in the [Zn(2-APTC)$_2$] group. Thus, glucose tolerance improved in the [Zn(2-APTC)$_2$] group (**Fig. 4**). As for HbA1c, a significant decrease was observed in the [Zn(2-APTC)$_2$] group, indicating the sustained hypoglycemic effect of [Zn(2-APTC)$_2$] (**Fig. 5**). The biochemical parameters were measured after administration. Significant increases in the BUN, AST, and ALT levels were observed in the [Zn(2-APTC)$_2$] group (**Fig. 5**). These results suggest that administration of [Zn(2-APTC)$_2$] may cause hepatic and renal dysfunction.

Next, the plasma leptin and insulin levels were measured. Although the plasma leptin levels tended to decrease in the [Zn(2-APTC)$_2$] group, the difference between the two groups was not significant. The plasma insulin levels did not differ significantly between the two groups (**Fig. 5**).

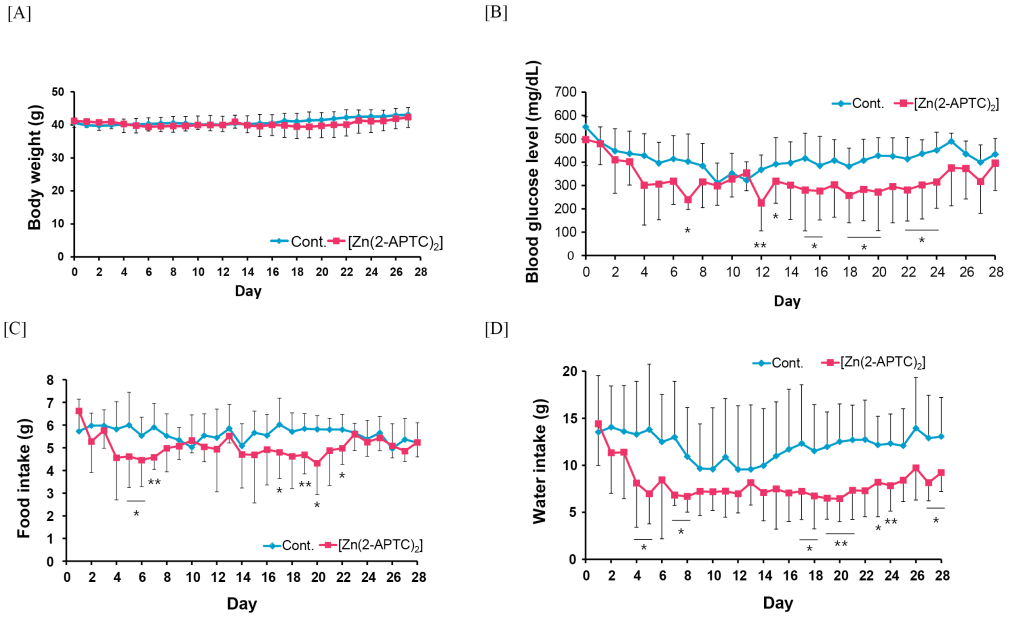


Fig. 3 Changes of body weight [A], Blood glucose level [B], food intake[C], and water intake[D] for 4 weeks.
Values are means ± SD for 7 mice. *Significance at P < 0.05 vs. control. **Significant at P < 0.01 vs. control.

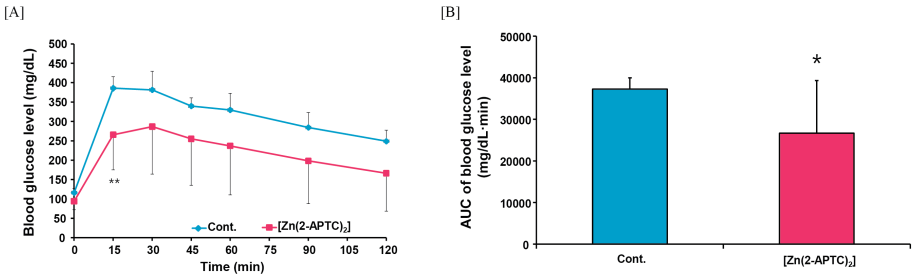


Fig. 4 Change of blood glucose level by OGTT [A] and AUC [B].
Values are means ± SD for 7 mice. *Significance at P < 0.05 vs. control. **Significant at P < 0.01 vs. control.

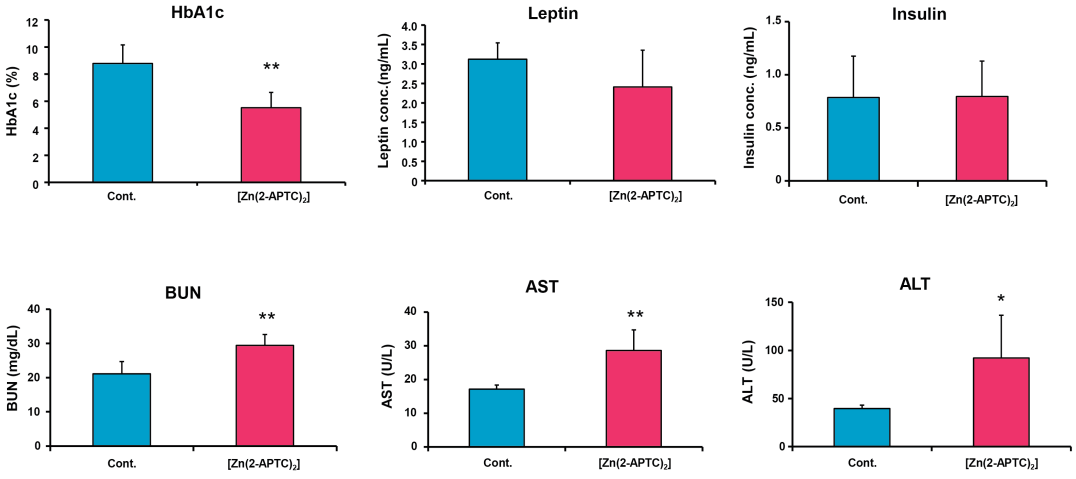


Fig. 5 HbA1c levels, biochemical parameters (BUN, AST, and ALT) in plasma and the plasma leptin and insulin levels after administration for 4 weeks.
Values are means ± SD for 7 mice. *Significance at P < 0.05 vs. control. **Significance at P < 0.01 vs. control.

Quantitative analysis of plasma and organs by ICP-MS showed that Zn levels were significantly increased in the liver, pancreas, and bones of the treated mice. Cu levels significantly decreased in the bone (**Table 2**).

From the evaluated results of the pancreatic and liver tissue sections of treated mice, there were no significant differences in the areas of the islets in the pancreas between the two groups (**Fig. 6**). In contrast, the number of mice with a low fat mass in the liver significantly increased in the [Zn(2-APTC)$_2$] group, indicating an improvement in fatty liver (**Fig.7, Table 3**).

Table 2 | Zn and Cu concentrations in organs after administration for 4 weeks. Values are means ± SD for 7 mice. *Significance at $P < 0.05$ vs. control.

	Cu (μg/mL or μg/g)		Zn (μg/mL or μg/g)	
	Cont.	[Zn(2-APTC)$_2$]	Cont.	[Zn(2-APTC)$_2$]
Liver	9.1 ± 1.4	10.4 ± 0.8	57.4 ± 7.1	96.5 ± 20.8*
Fat	0.2 ± 0.0	0.2 ± 0.1	31.4 ± 9.0	30.8 ± 10.6
Kidney	9.0 ± 0.7	8.4 ± 0.9	64.0 ± 7.8	68.7 ± 22.5
Plasma	0.7 ± 0.1	0.6 ± 0.1	8.3 ± 2.8	5.7 ± 2.4
Pancreas	3.4 ± 1.4	4.1 ± 1.1	113.3 ± 19.9	242.4 ± 84.6*
Spleen	2.3 ± 0.3	2.2 ± 0.2	70.0 ± 11.2	66.1 ± 14.8
Muscle	1.9 ± 0.4	1.4 ± 0.2	43.5 ± 11.7	52.7 ± 18.7
Bone	0.5 ± 0.1	0.3 ± 0.1*	118.6 ± 8.2	191.7 ± 51.2*

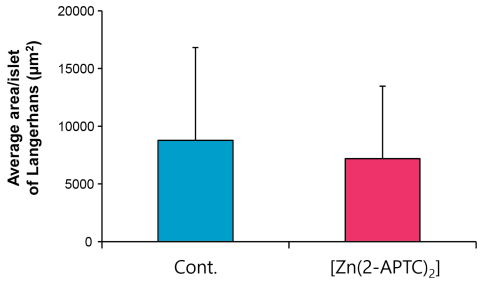


Fig. 6 | Areas of islets of Langerhans in pancreas. Values are means ± SD for 7 mice.

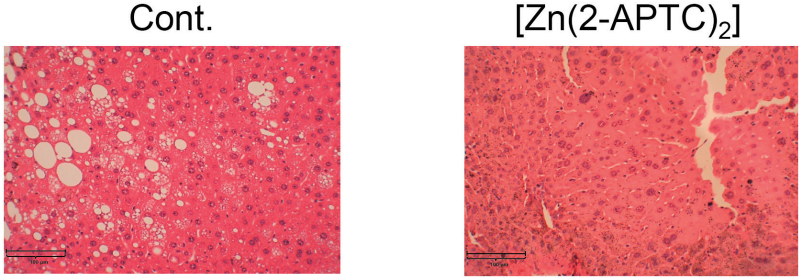


Fig. 7 | Pictures of liver tissue (×200)

Table 3 Hepatic morphology of fatty liver in 9-week-old control KK-A^y mice and [Zn(2-APTC)$_2$] adminisitrated KK-A^y mice (×200).
*Significance at $P < 0.05$ vs. control.
The evaluation score: 0 for 0-10%, +1 for 10-30%, +2 for 30-50%, and +3 for more than 50%.

	0	+1	+2	+3	n
Cont.	1	1	2	3	7
[Zn(2-APTC)$_2$]*	2	4	1	0	7

Discussion

Based on the results of the *in vitro* FFA test, [Cu(2-APTC)$_2$] and [Zn(2-APTC)$_2$] showed high insulin-like activity. In our previous study, we reported that Cu(II)-picolinate complex showed the *in vivo* antidiabetic effect [12]. In this study, the other Cu(II) complex, [Cu(2-APTC)$_2$], exhibited the stronger *in vitro* insulin-like activity than Cu^{2+} ion. On the other hand, there are few studies of antidiabetic effect of Ni(II) compounds, and we additionally evaluated the Ni^{2+} ion and Ni(II) complex, [Ni(2-APTC)$_2$], and revealed that Ni did not show any activity to inhibit FFA release like insulin. Although the *in vitro* insulin-like activity of [Cu(2-APTC)$_2$] was considerably potent, the intestinal absorption of [Cu(2-APTC)$_2$] was lower than that of [Zn(2-APTC)$_2$]. Therefore, we continued to evaluate the *in vivo* antidiabetic effects of [Zn(2-APTC)$_2$] in detail. In the present study, the [Zn(2-APTC)$_2$] group showed a decrease in blood glucose, water intake, and HbA1c, while the OGTT indicated that the increase in blood glucose was suppressed (**Fig. 3-5**). These results indicate that [Zn(2-APTC)$_2$] has antidiabetic effects, such as lowering blood glucose, improving in HbA1c, and ameliorating glucose tolerance. A previous study evaluating the antidiabetic effects of a complex with 4-coordinated thiosemicarbazone as the ligand showed stronger hypoglycemic effects [23] than the complex used in this study. Changing the physicochemical properties of the ligand, such as the molecular weight and lipophilicity, may enhance its antidiabetic effect.

Plasma leptin and insulin levels are generally elevated because diabetic patients often exhibit leptin and insulin resistance [24]. In the present study, improvements in leptin and insulin resistance were not involved in the antidiabetic effects of [Zn(2-APTC)$_2$], as no significant difference was found between the two groups. Since a previous study on the antidiabetic effects of the thiosemicarbazone complex suggested its involvement in leptin resistance [23], it has been suggested that contributions to the antidiabetic effect may differ among thiosemicarbazone compounds. The results the ICP-MS analysis for [Zn(2-APTC)$_2$] showed that Zn mainly accumulated in the liver, pancreas, and bone. Previous studies have suggested that Zn complexes mainly affect the pancreas, muscles, and fat, and may be involved in promoting insulin secretion in the pancreas, affecting adenosine monophosphate-activated protein kinase (AMPK), activating the insulin cascade, and promoting adiponectin secretion in fat [9, 25-28]. Based on the results of this study, we hypothesized that the antidiabetic effect of [Zn(2-APTC)$_2$] was due to the action of Zn in the pancreas. However, insulin also reduces liver gluconeogenesis and promotes glycogen synthesis [29]. Thus, it is highly likely that [Zn(2-APTC)$_2$], which exhibits insulin-like effects, also exhibits antidiabetic effects owing to its involvement in the inhibition of gluconeogenesis and promotion of glycogen synthesis in the liver.

Plasma BUN, ALT, and AST levels were higher in the [Zn(2-APTC)$_2$] group than in the control group. This indicates that [Zn(2-APTC)$_2$] may have undesirable effects on renal and hepatic functions.

[Zn(2-APTC)$_2$] may exert its antidiabetic effects through the inhibition of gluconeogenesis in the liver and the induction of glycogen synthesis. Metformin and buformin are used worldwide as oral hypoglycemic agents that inhibit liver gluconeogenesis [30]. However, these drugs are known to cause hepatic dysfunction [31]. It has been suggested that [Zn(2-APTC)$_2$] has antidiabetic effects in the liver; simultaneously, it may also cause liver dysfunction.

In this study, [Zn(2-APTC)$_2$] showed excellent antidiabetic effects such as lowering blood glucose, improving glucose tolerance, and ameliorating fatty liver after 28 d of continuous oral administration. These results suggest that Zn(II) complexes, which have thiosemicarbazone derivatives as ligands, are potential candidates for metal complexes with antidiabetic activity. However, the results also showed side effects of [Zn(2-APTC)$_2$] in the liver and kidneys. Based on these findings, it is necessary to evaluate the effects and toxicity of this compound in the future.

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Author Contributions

Chihiro Tanaka: Investigation. Yuki Naito: Investigation, Visualization, Draft preparation. Yutaka Yoshikawa: Investigation, Draft preparation, Supervision. Hiroyuki Yasui: Supervision, Writing- reviewing and editing.

References

[1] Cho NH, Whiting D, Guariguata L, Montoya PA, Forouhi P, Hambleton I, Li R, Majeed A, Mbanya JC, Motala A, Narayan VKM, Ramachandran A, Rathmann W, Roglic G, Shaw J, Silink M, Williams DRR, Zhang P. *IDF Diabetes ATLAS 6th Edition 2014 UPDATE*. International Diabetes Federation; 2014. Available from: https://diabetesatlas.org/idfawp/resource-files/2010/07/IDF_diabetes_atlas_sixth_edition_en.pdf

[2] Artasensi A, Pedretti A, Vistoli G, Fumagalli L. Type 2 diabetes mellitus: A review of multi-target drugs. *Molecules* 2020; 25(8): 1987.

[3] Araki E, Goto A, Kondo T, Noda M, Noto H, Origasa H, Osawa H, Taguchi A, Tanizawa Y, Tobe K, Yoshioka N. Japanese Clinical Practice Guideline for Diabetes 2019. *Diabetol Int*. 2020; 11(3): 165-223.

[4] Johnson-Rabbett B, Seaquist ER. Hypoglycemia in diabetes: The dark side of diabetes treatment. A patient-centered review. *J Diabetes*. 2019; 11(9): 711-718.

[5] Chasapis CT, Ntoupa PA, Spiliopoulou CA, Stefanidou ME. Recent aspects of the effects of zinc on human health. *Arch Toxicol*. 2020; 94(5): 1443-1460.

[6] Coulston L, Dandona P. Insulin-like effect of zinc on adipocytes. *Diabetes*. 1980; 29(8): 665-667.

[7] Chen MD, Liou SJ, Lin PY, Yang VC, Alexander PS, Lin WH. Effects of zinc supplementation on the plasma glucose level and insulin activity in genetically obese (ob/ob) mice. *Biol Trace Elem Res*. 61(3): 303-311.

[8] Oke IM, Ramorobi LM, Mashele SS, Bonnet SL, Makhafola TJ, Eze KC, Noreljaleel AEM, Chukwuma CI. Vanillic acid-Zn(II) complex: a novel complex with antihyperglycaemic and anti-oxidative activity. *J Pharm Pharmacol*. 2021; 73(12): 1703-1714.

[9] Yoshikawa Y, Murayama A, Adachi Y, Sakurai H, Yasui H. Challenge of studies on the development of new Zn complexes ($Zn(opt)_2$) to treat diabetes mellitus. *Metallomics*. 2011; 3(7): 686-692.

[10] Yoshikawa Y, Ueda E, Kawabe K, Miyake H, Sakurai H, Kojima Y. New insulin-mimetic zinc (II) complexes; Bis-maltolato zinc (II) and bis-2-hydroxypyridine-N-oxido zinc (II) with $Zn(O_4)$ coordination mode. *Chem. Lett*. 2000; 29(8): 874-875.

[11] Nishiguchi T, Yoshikawa Y, Yasui H. Investigating the target organs of novel anti-diabetic zinc complexes with organo-selenium ligands. *J Inorg Biochem*. 2018; 185: 103-112.

[12] Yasumatsu N, Yoshikawa Y, Adachi Y, Sakurai H. Antidiabetic copper(II)-picolinate: Impact of the first transition metal in the metallopicolinate complexes. *Bioorg Med Chem*. 2007; 15(14): 4917-4922.

[13] Sakthivel RV, Sankudevan P, Vennila P, Venkatesh G, Kaya S, Serdaroğlu G. Experimental and theoretical analysis of molecular structure, vibrational spectra and biological properties of the new Co(II), Ni(II) and Cu(II) Schiff base metal complexes. *J Mol Structure*. 2021; 1233: 130097.

[14] Rogolino D, Bacchi A, De Luca L, Rispoli G, Sechi M, Stevaert A, Naesens L, Carcelli M. Investigation of the salicylaldehyde thiosemicarbazone scaffold for inhibition of influenza virus PA endonuclease. *J Biol Inorg Chem*. 2015; 20(7): 1109-1121.

[15] Pelosi G. Thiosemicarbazone metal complexes: From structure to activity. *The Open Crystallography Journal*. 2010; 3: 16-28.

[16] Parul N, Subhangkar N, Arun M. Antimicrobial activity of different thiosemicarbazone compounds against microbial pathogens. *Int Res J Pharm*. 2012; 3(5): 350-363.

[17] Li MX, Zhang LZ, Yang M, Niu JY, Zhou J. Synthesis, crystal structures, in vitro biological evaluation of zinc(II) and bismuth(III) complexes of 2-acetylpyrazine *N*(4)-phenylthiosemicarbazone. *Bioorg Med Chem Lett*. 2012; 22(7): 2418-2423.

[18] Li MX, Zhang D, Zhang LZ, Niu JY, Ji BD. Diorganotin(IV) complexes with 2-benzoylpyridine and 2-acetylpyrazine *N*(4)-phenylthiosemicarbazones: Synthesis, crystal structures. *J Organometal Chem*. 2011; 696(4): 852-858.

[19] Li MX, Chen CL, Zhang D, Niu JY, Ji BS. Mn(II), Co(II) and Zn(II) complexes with heterocyclic substituted thiosemicarbazones: Synthesis, characterization, X-ray crystal structures and antitumor comparison. *Eur J Med Chem*. 2010; 45(7): 3169-3177.

[20] Joseph M, Kuriakose M, Kurup MRP, Suresh E, Kishore A, Bhat SG. Structural, antimicrobial and spectral studies of copper(II) complexes of 2-benzoylpyridine *N*(4)-phenyl thiosemicarbazone. *Polyhedron*. 2006; 25(1): 61-70.

[21] Li MX, Chen CL, Ling CS, Zhou J, Ji BS, Wu YJ, Niu JY. Cytotoxicity and structure-activity relationships of four α-N=heterocyclic thiosmicarbazone derivatives crystal structure of 2-acetylpyrazine thiosemicarbazone. *Bioorg Med Chem Lett*. 2009; 19(10): 2704-2706.

[22] Nakai M, Watanabe H, Fujiwara C, Kakegawa H, Satoh T, Takada J, Matsushita R, Sakurai H. Mechanism on Insulin-Like Action of Vanadyl Sulfate: Studies on Interaction between Rat Adipocytes and Vanadium Compounds. *Biol Pharm Bull*. 1995; 18(5): 719-725.

[23] Kadowaki S, Munekane M, Kitamura Y, Hiromura M, Kamino S, Yoshikawa Y, Saji H, Enomoto S. Development of new zinc dithiosemicarbazone complex for use as oral antidiabetic agent. *Biol Trace Elem Res*. 2013; 154(1): 111-119.

[24] Huang J, Peng X, Dong K, Tao J, Yang Y. T The Association Between Insulin Resistance, Leptin, and Resistin and Diabetic Nephropathy in Type 2 Diabetes Mellitus Patients with Different Body Mass Indexes. *Diabetes Metab Syndr Obes*. 2021; 14: 2357-2365.

[25] Moroki T, Yoshikawa Y, Yoshizawa K, Tsubura A, Yasui H. Morphological analysis of the pancreas and liver in diabetic KK-A^y mice treated with zinc and oxovanadium complexes. *Metallomics*. 2014; 6(9): 1632-1638.

[26] Zhang C, Lu X, Tan Y, Li B, Miao X, Jin L, Zhang X, Miao L, Li X, Cai L. Diabetes-induced hepatic pathogenic damage, inflammation, oxidative stress, and insulin resistance was exacerbated in zinc deficient mouse model. *PLoS One*. 2012; 7(12): e49257.

[27] Jansen J, Karges W, Rink L. Zinc and Diabetes – clinical links and molecular mechanisms. *J Nutr Biochem*. 2009; 20(6): 399-417.

[28] Wiernsperger N, Rapin J. Trace elements in glucometabolic disorders: an update. *Diabetol Metab Synd*. 2010; 2: 70.

[29] Hatting M, Tavares CDJ, Sharabi K, Rines AK, Puigserver P. Insulin regulation of gluconeogenesis. *Ann N Y Acad Sci*. 2018; 1411(1): 21-35.

[30] de Souza Silva FM, da Silva MHRA, Bracht A, Eller GJ, Constantin RP, Yamamoto NS. Effects of metformin on glucose metabolism of perfused rat livers. *Mol Cell Biochem*. 2010; 340(1-2): 283-289.

[31] Lalau J. Lactic acidosis induced by metformin: incidence, management and prevention. *Drug Saf*. 2010; 33(9): 727-740.