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Regular article

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 S₂N₄-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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Keywords: Type 2 diabetes mellitus, thiosemicarbazone, insulin, KK-A^y mice, zinc complex

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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 S₂N₄ 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.



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(ClO₄)₂·6H₂O), nickel perchlorate hexahydrate (Ni(ClO₄)₂·6H₂O), sodium acetate (CH₃COONa), zinc (II) sulfate heptahydrate (ZnSO₄·7H₂O), copper (II) sulfate pentahydrate (CuSO₄·5H₂O), nickel (II) sulfate hexahydrate (NiSO4·6H2O), NEFA-C Test Wako, glucose, sodium chloride (NaCl), calcium chloride (CaCl2), magnesium sulfate (MgSO₄) potassium chloride (KCl), potassium dihydrogen phosphate (KH₂PO₄), sodium bicarbonate (NaHCO₃), 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(ClO₄)₂·6H₂O) was purchased from KANTO CHEMICAL CO., INC. (Kyoto, Japan). Acetic acid (CH₃COOH), 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 lowresolution 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 CH₃COOH, 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 CH₃COONa (0.4 mmol), 2-APTC (0.4 mmol), and Zn(ClO₄)₂·6H₂O (0.2 mmol) was dissolved in MeOH. The Zn(ClO₄)₂·6H₂O solution was stirred and the 2-ATPC solution with CH₃COONa 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)

CH₃COONa (0.4 mmol), 2-APTC (0.4 mmol) and Ni(ClO₄)₂·6H₂O (0.2 mmol) were each dissolved in MeOH. The Ni(ClO₄)₂·6H₂O solution was stirred and the 2-ATPC solution with CH₃COONa 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 CaCl₂, 1.20 mM MgSO₄, 4.75 mM KCl, 1.20 mM KH₂PO₄, 24 mM NaHCO₃, 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 × 10⁶ 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. ZnSO₄·7H₂O, CuSO₄·5H₂O, and NiSO₄·6H₂O 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 (IC₅₀), 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 $[Cu(2-APTC)_2]$ and $[Zn(2-APTC)_2]$ 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 CuSO₄·5H₂O and ZnSO₄·7H₂O, 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% HNO₃. 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-APTC)₂]

Nine-week-old KK-A^y mice were divided into the control (Cont. group, n=7) and treatment groups ($[Zn(2-APTC)_2]$ group, n=7). The Cont. group was orally administered PEG 400 and the $[Zn(2-APTC)_2]$ group was orally administered 15 mg Zn/kg BW of $[Zn(2-APTC)_2]$ 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 $[Zn(2-APTC)_2]$ 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 $(C_{26}H_{24}N_{10}S_2Cu\cdot0.5H_2O)$, 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 $(C_{26}H_{24}N_{10}S_2Ni\cdot0.7H_2O)$, 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 dosedependent inhibitory effects on free fatty acid release, that is, an insulin-like effect. In particular, the IC₅₀ values of $[Zn(2-APTC)_2]$ and $[Cu(2-APTC)_2]$ were $16.9 \pm 6.2 \,\mu$ M and $11.3 \pm 3.6 \,\mu$ M, respectively, and their inhibitory effects were seen at much lower concentrations than that of $ZnSO_4.7H_2O$. This insulin-like effect was also observed for NiSO₄.6H₂O used as an indicator of Ni ion, but the effect was far weaker than those of $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$. However, there was no insulin-like effect of ligand 2-APTC.

Table 1	Result of insulin-like effect. Estimated IC ₅₀ values of	Compounds	IC ₅₀ (μM)	
	each compound for FFA release from rat adipocytes	ZnSO ₄ ·7H ₂ O	531 ± 52	_
	Data are expressed as the means \pm SD for 3	CuSO ₄ ·5H ₂ O	298 ± 67	
	independent experiments. **Significant at $P < 0.01$ vs.	NiSO ₄ .6H ₂ O	Low activity	
	ZnSO ₄ ·7H ₂ O. ^{##} Significant at $P < 0.01$ vs. CuSO ₄ ·5H ₂ O.	[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 $CuSO_4$ ·5H₂O. 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 $ZnSO_4$ ·7H₂O (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.



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**).













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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**).

			at 1 < 0.05 V3. CO	
	Cu (µg/i	mL or μg/g)	Zn (µg/n	nL 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	$242.4\pm84.6^*$
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	$0.3\pm0.1^*$	118.6 ± 8.2	$191.7 \pm 51.2^{*}$

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



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



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

 $[Zn(2-APTC)_2]^*$

2

Table 3	Hepatic m [Zn(2-APT(*Significar The evalua more than	orphology C) ₂] admini nce at P < 0 ation score: 1 50%.	of fatty liver in sitrated KK-A ^y n 0.05 vs. control. : 0 for 0-10%, + ⁻	9-week-old co nice (×200). I for 10-30%, ·	ontrol KK-A' m +2 for 30-50%	iice and , and +3 for
		0	+1	+2	+3	n
Co	nt.	1	1	2	3	7

1

0

7

4

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 Ni²⁺ 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, 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.

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Regular article

Dietary iron supplementation reverses the effects of iron deficiency on bone formation markers in iron-deficient rats

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Abstract

Iron plays important roles in bone health as it is required as a cofactor for renal 25-hydroxyvitamin D-1 α -hydroxylase, which is involved in the production of 1,25-dihydroxyvitamin D₃ (active vitamin D), and regulates the expression of proteins involved bone formation. Herein, we investigated the effects of dietary iron supplementation on bone formation in iron-deficient rats. Male Wistar rats were fed a control (n = 12) or iron-deficient diet (n = 18) for 30 days, after which 6 rats in the iron-deficient group were switched to the control diet for 14 days. At the end of the 30-day period, hemoglobin, liver iron, serum 1,25-dihydroxyvitamin D₃, and serum osteocalcin concentrations, and femoral bone mineral density (BMD) were found to be significantly lower in iron-deficient rats than that in control rats. Following dietary iron supplementation, hemoglobin and liver iron concentrations elevated significantly in iron-deficient rats; however, they were not restored to the normal levels. Additionally, dietary iron supplementation increased serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations and femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats, which were downregulated by iron deficiency, to levels similar to that in control rats. However, femoral BMD was not recovered with dietary iron supplementation. These results suggest that iron deficiency-induced downregulation of bone formation can potentially be reversed by dietary iron supplementation. Furthermore, elevated serum 1,25-dihydroxyvitamin D₃ concentrations due to dietary iron supplementation could be responsible for increased expression of bone formation markers in iron-deficient rats.

Keywords: iron deficiency, dietary iron supplementation, bone formation, 1,25-dihydroxyvitamin D₃, rats

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

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Introduction

Iron deficiency is one of the most common nutritional deficiencies worldwide, and reportedly affects bone health. One of the mechanisms through which iron participates in bone formation is through its role in the activation of vitamin D. Renal 25-hydroxyvitamin D-1 α -hydroxylase (1 α -hydroxylase), an enzyme that used iron as a cofactor, converts 25-hydroxyvitamin D to its active form vitamin D [1]. We previously demonstrated that dietary iron deficiency diminishes renal 1 α -hydroxylase activity, which consequently leads to a decrease in serum 1,25-dihydroxyvitamin D₃ concentration in rats [2]. Given the role of 1,25-dihydroxyvitamin D₃ in bone metabolism, lower



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serum 1,25-dihydroxyvitamin D3 concentration could potentially result in decreased bone formation in iron-deficient rats.

Moreover, previous studies have investigated the relationship between dietary iron and bone mineral density (BMD). In human studies, dietary iron was found to be positively associated with BMD in postmenopausal women [3, 4]. Medeiros *et al.* reported that iron deficiency decreases BMD, bone mineral content, and bone strength in female weanling rats [5]. These findings were consistent with the results of our previous studies with male weanling rats [6, 7]. Furthermore, we previously showed that an iron-deficient diet causes a decrease in serum osteocalcin concentration in rats [6, 7]. In addition, dietary iron deficiency decreases serum procollagen type I N-terminal propeptide (P1NP) concentration in rats [8]. Therefore, we speculated that dietary iron deficiency inhibits osteoblastic bone formation, which could potentially account for diminished BMD in iron-deficient rats.

It has been speculated that the physiological changes caused by an iron-deficient diet can be restored by re-addition of iron into the diet. Consistently, it is speculated that the decrease in bone formation due to iron deficiency can be improved following recovery from iron deficiency. Therefore, in the present study, we investigated the effects of dietary iron supplementation on bone formation in iron-deficient rats.

Materials and Methods

Ethical approval

This study was approved by the Tokyo University of Agriculture Animal Use Committee (approval number 280070) and performed in accordance with the university guidelines for the care and use of laboratory animals.

Experimental design

Thirty 3-week-old male Wistar rats (Clea Japan, Tokyo, Japan) were individually housed in stainless-steel cages at 22 °C in a 12-hour light-dark cycle. Throughout the study period, the rats were fed a control diet or an iron-deficient diet and were given free access to deionized water. Experimental diets were prepared according to the AIN-93G formula [9]. The iron-deficient diet was prepared from a basal diet by adding iron-free AIN-93G mineral mixture instead of AIN-93G mineral mixture. Iron content of the control and iron-deficient diets were 54.5 µg/g and 8.9 µg/g, respectively.

Iron-deficient rats were fed an iron-deficient diet for 30 days. Briefly, rats $(58.6 \pm 2.9 \text{ g})$ were divided into the control (C0, n = 12) and iron-deficient (D0, n = 18) groups. The D0 group was given free access to an iron-deficient diet, and the C0 group was pair-fed the control diet at the mean intake of the D0 group for 30 days. On day 30, six rats from each group were sacrificed under anesthesia by intraperitoneal injection of anesthetic mixture (medetomidine, midazolam, and butorphanol), and blood, liver, and the left femur were collected for further analysis.

After the initial 30 day feeding period, rats in the D0 group were divided into the iron-deficient (D, n = 6) and recovery (R, n = 6) groups. Rats in D group were provided free access to an iron-deficient diet for another 14 days. Whereas, rats in the R and C0 groups (C, n = 6) were pair-fed a control diet at the mean intake of the D group. After 14 days, all rats were sacrificed under anesthesia, and blood, liver, and the left and right femurs were collected for further analysis. The hearts were removed and weighed to assess cardiac enlargement, a sign of iron-deficiency anemia.

Assessment of serum levels of bone formation markers and 1,25-dihydroxyvitamin D₃

Hemoglobin concentration in blood samples was measured using the Hemoglobin B-test Wako (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Additionally, blood samples were centrifuged at 1,500 × g for 15 min, and the supernatants were collected as serum samples to analyze the levels of osteocalcin, a bone formation marker and 1,25-dihydroxyvitamin D₃. Serum samples were stored at -80 °C until analysis. Serum 1,25-dihydroxyvitamin D₃ concentration was measured using the 1,25(OH)₂ vitamin D ELISA kit (Immundiagnostik AG, Bensheim, Germany). Serum osteocalcin concentration was measured using the Rat Osteocalcin (BGP) ELISA system (GE Healthcare Japan Corporation, Tokyo, Japan).

Assessment of iron content in the liver

Briefly, liver samples were flushed with ice-cold 0.9% NaCl through the portal vein. One gram of liver samples were dried at 100 °C for 24 hours. For measurement of iron, dried liver samples were ashed in a muffle furnace at 550 °C for 48 hours, and the minerals were diluted with 1 mol/L HCl solution. Subsequently, the iron content was analyzed using an atomic absorption spectrophotometer (ZA3300; Hitachi, Ltd., Tokyo, Japan).

Assessment of BMD

Following collection, left femur samples were placed in 70% ethanol at 4 °C until analysis, and dried at 60 °C overnight. Subsequently, BMD of the femur was measured using dual-energy X-ray absorptiometry (DXA; DSC-600EX, Hitachi Aloka Medical, Ltd., Tokyo, Japan).

Real-time PCR to assess mRNA levels of osteocalcin and collagen type I alpha 1 chain

Right femur samples were removed and stored at -80 °C until further analysis. The femur samples were homogenized in TRI Reagent (Molecular Research Center Inc., OH, USA) using ceramic bead-containing tubes and a Precellys homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) with two cycles of 7,500 rpm for 20 seconds. Total RNA was isolated from the cell lysate using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). The following primers were used for analysis: osteocalcin (*Bglap*), forward, 5'-GAGCTAGCGGACCACATTGG-3' and reverse, 5'-CCTAAACGGTGGTGCCATAGA-3'; collagen type I alpha 1 chain (*Col1a1*), forward, 5'-TTCACCTACAGCACGCTTGTG-3' and reverse, 5'-GATGACTGTCTTGCCCCAAGTT-3'; β-actin (*Actb*), forward, 5'-AGCCATGTAGCGTAGCCATCCA-3' and reverse, 5'-TCTCCGGAGTCCATCACAATG-3' [10, 11]. *Bglap* and *Col1a1* mRNA expression was normalized to that of the housekeeping gene, *Actb*.

Statistical analysis

Results are expressed as the mean \pm standard error for each group (n = 6 rats/group). Statistical analyses were performed using the SPSS Statistics ver.23 (IBM, Armonk, NY, USA). Homogeneity of variance between the C0 and D0 groups was assessed using the F-test, and an independent t-test was used to determine significant differences between the C0 and D0 groups. Oneway analysis of variance followed by the least significant difference test was performed to evaluate significant differences among the C, D, and R groups. Differences were considered statistically significant at p < 0.05.

Results

Effects of dietary iron deficiency on body weight, iron status, serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations, and femoral BMD

There was no significant difference in final body weight between the C0 and D0 groups (**Table 1**). However, heart weight was significantly higher in the D0 group than that in the C0 group, suggesting cardiac enlargement. Consistently, hemoglobin and liver iron concentrations were significantly lower in the D0 group than that in the C0 group. Additionally, serum 1,25-dihydroxyvitamin

Table 1.Body weight, indicators of iron deficiency, serum 1,25-dihydroxyvitamin D₃ and
osteocalcin concentrations, and BMD of the femur in the C0 and D0 groups.

	C0	D0
Final body weight (g)	209.5 ± 2.5	199.6 ± 4.6
Heart weight (g/100 g body weight)	0.32 ± 0.01	$0.56\pm0.02^*$
Hemoglobin (g/dL)	11.41 ± 0.43	$3.59\pm0.10^*$
Liver iron (μg/g dry weight)	402.8 ± 37.6	$151.4\pm7.6^*$
Serum 1,25-dihydroxyvitamin D ₃ (pg/mL)	112.2 ± 4.4	$81.0\pm6.5^*$
Serum osteocalcin (ng/mL)	91.8 ± 2.6	$80.8\pm3.1^*$
Femur BMD (mg/cm ²)	108.4 ± 1.9	$102.1 \pm 1.6^{*}$

C0, control group; D0, iron-deficient group; BMD, bone mineral density. Data are presented as the mean \pm standard error (SE) for each group (n = 6).

*Significant difference compared to the C0 group, *p* < 0.05.

 D_3 and osteocalcin concentrations and femur BMD were significantly lower in the D0 group that than in the C0 group. These findings suggest that iron deficiency affects bone health though regulation of 1,25-dihydroxyvitamin D_3 and osteocalcin.

Effects of dietary iron supplementation on body weight and iron status of iron-deficient rats

There was no significant difference in final body weight among the C, D, and R groups (**Table 2**). However, heart weight was significantly higher in the D group than that in the C group and significantly lower in the R group than in the D group. Moreover, hemoglobin and liver iron concentrations were significantly lower in the D group than that in the C group and were significantly higher in the R group than that in the D group. These findings suggest that iron supplementation in iron-deficient rats cannot completely reverse the effects of iron deficiency, namely cardiac enlargement and anemia.

	С	D	R
Final body weight (g)	251.7 ± 1.5	230.3 ± 10.9	232.5 ± 4.2
Heart weight (g/100 g body weight)	$0.29\pm0.01^{\circ}$	0.62 ± 0.03^{a}	$0.43\pm0.02^{\rm b}$
Hemoglobin (g/dL)	$14.0\pm0.5^{\text{a}}$	$3.2 \pm 0.2^{\circ}$	$11.2\pm0.2^{\rm b}$
Liver iron (µg/g dry weight)	$444.1\pm20.8^{\text{a}}$	$161.9 \pm 8.9^{\circ}$	$210.4\pm7.3^{\rm b}$

Table 2. | Body weight and indicators of iron deficiency in the control, iron-deficiency, and recovery groups.

C, control group; D, iron-deficient group; R, recovery group. Data are presented as the mean ± SE for each group of 6 rats.

^{a,b,c}The different letters denote significant differences, p < 0.05.

Effects of dietary iron supplementation on serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations, femoral BMD, and femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats

Serum 1,25-dihydroxyvitamin D₃ concentrations were significantly lower in the D group than that in the C group and significantly higher in the R group than that in the D group (**Figure 1**). Serum osteocalcin concentration and mRNA expression of *Bglap* and *Col1a1* were significantly lower in the D group than that in the C group and were significantly higher in the R group than in the D group (**Figure 2**). Additionally, consistent with our expectations, femoral BMD was significantly lower in the D group than that in the C group. However, in contrast with the expression of bone formation markers, femoral BMD did not differ between the D and R groups. There was no significant difference in femoral BMD between the C and R groups. These findings suggest that although iron supplementation in iron-deficient rats can reverse the effect of iron deficiency on bone formation markers, but not completely reverse femoral BMD, at least in the recovery period studied herein.







Figure 2. (a) Serum osteocalcin concentration, (b) bone mineral density (BMD) of the femur, (c) *Bglap* mRNA expression, and (d) *Col1a1* mRNA expression in the control (C), iron-deficient (D), and recovery (R) groups. Data are presented as the mean \pm SE for each group (n = 6 rats/group).^{a,b,c}The different letters denote significant differences, p < 0.05.

Discussion

It has previously been reported that dietary iron deficiency can resulted in decreased body weight gain due to reduced food intake in rats [5-7]. Thus, an iron-deficient diet might result in iron deficiency along with other nutrient deficiencies, such as calcium and vitamin D deficiency, which would impact bone health. Furthermore, a decrease in body weight gain is thought to result in lower BMD. In the present study, we investigated the effects of iron deficiency and recovery under paired feeding conditions. Herein, an iron-deficient diet for a duration of 30 days followed by dietary iron supplementation for 14 days did not affect body weight. Therefore, in the present study, we could examine the effects of iron deficiency and iron supplementation on bone formation without the added effects of altered food intake or body weight gain.

Knutson *et al.* reported marked reductions in hemoglobin concentration, liver iron concentration, and percentage of transferrin saturation in rats fed an iron-deficient diet for 35 days, indicating severe anemia [12]. Consistently, in the present study, 30 days of an iron-deficient diet markedly reduced hemoglobin and liver iron concentrations in iron-deficient rats. Further, we previously reported that hemoglobin concentration in iron-deficient rats was reduced to approximately 25% of that in pair-fed control rats [7]. In the present study, the hemoglobin concentration in the D0 group was significantly reduced to approximately 31% of that in the C0 group. Thus, consistent with previous studies [7, 12], we were able to produce a severe iron-deficiency anemia model through a 30 day iron-deficient diet.

In the present study, dietary iron supplementation increased hemoglobin concentration in iron-deficient rats; however, the hemoglobin concentration in the R group was not restored to that in the C group. Additionally, liver iron concentration, which was decreased by iron deficiency, increased slightly following dietary iron supplementation. Consistent with our findings, Tsao *et al.* previously reported that although the decreased hemoglobin concentration caused by an iron-deficient diet increased following 2-week iron supplementation, the iron-supplemented group still had significantly lower hemoglobin concentration

when compared to the control group [13]. Furthermore, rat red blood cells have been reported to have a life span of only 60 days [14]. Therefore, taking into account the results of hemoglobin concentration and iron storage in the liver, the iron status of the R group did not recover to the normal range following iron supplementation. As a results, dietary iron supplementation in iron-deficient rats could not completely reverse the effects of iron deficiency, namely heart enlargement and anemia in this study.

Previous studies have reported that dietary iron deficiency decreases femoral BMD in rats [5-7], and similar results were observed in the present study. However, femoral BMD did not differ between the D and R groups. Also, there was no significant difference in femoral BMD between the C and R groups. These results suggest that dietary iron supplementation in iron-deficient rats may restore femoral BMD, but not fully recovered, at least in the recovery period of this study. We speculate that dietary iron supplementation can increase femoral BMD in iron-deficient rats when the supplementation period is prolonged. Hence, the effects of iron status on femoral BMD could not be fully understood in this study, and further studies should be study the same, with an extended period of dietary iron supplementation and/or different doses of iron supplementation.

The decrease in BMD induced by iron deficiency may be attributed to decreased bone formation. Previously, we reported that dietary iron deficiency decreased the levels of serum osteocalcin concentration [6, 7]. Díaz-Castro *et al.* reported that dietary iron deficiency decreased serum P1NP concentration, a marker of bone formation [8]. In addition, bone histomorphometric parameters for bone formation, such as bone formation rate and osteoblast surface, were found to be decreased in iron-deficient rats [2, 7]. In the present study, dietary iron deficiency decreased serum osteocalcin concentration, suggesting that a decrease in bone formation in iron-deficient rats. Moreover, serum osteocalcin concentration in iron-deficient rats was recovered following dietary iron supplementation and was similar to that in the control rats. These results suggested that dietary iron supplementation increased bone formation, which was suppressed by iron deficiency, although the iron status was not fully restored to the normal range.

Bone matrix proteins such as type I collagen and osteocalcin are used as markers of the osteoblast differentiation. In general, *Col1a1* are early marker of osteoblast differentiation, while *Bglap* appear late, concomitantly with mineralization [15]. In the present study, femoral mRNA expression of *Bglap* and *Col1a1* was significantly decreased in iron-deficient rats compared to that in control rats. Furthermore, dietary iron supplementation increased femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats. We previously showed that iron deficiency downregulated *Bglap* and *Col1a1* mRNA expression in rats, along with downregulation of *Runx2* mRNA expression was observed [2]. Given that Runx2 regulates the expression of several osteoblastic genes, including *Bglap* and *Col1a1* [16], these results suggested that diminished Runx2 expression due to iron deficiency resulted in decreased bone formation. In the present study, we did not measure femoral *Runx2* mRNA expression. Consequently, decreased expression of osteoblastic genes expression due to iron deficiency may be recovered by iron supplementation, which would also regulate changes in bone formation. In addition, femoral *Bglap* mRNA expression was relatively higher in the R group than in the C group. Previous study on recovery from zinc deficiency showed that plasma zinc concentration was relatively higher in the recovery rats than in the control rats [17]. Therefore, the *Bglap* mRNA expression, which was decreased by iron deficiency, was thought to be overexpressed by restoring iron.

Our previous studies showed that iron deficiency decreased serum 1,25-dihydroxyvitamin D₃ concentrations in rats [2, 7]. Renal 1 α -hydroxylase converts 25-hydroxyvitamin D₃ to 1,25-dihydroxyvitamin D₃, and used iron as a cofactor [1]. Therefore, iron deficiency is thought to decrease bone formation owing to a decline in vitamin D activation. Consistent with our previous studies [2, 7], in the present study, serum 1,25-dihydroxyvitamin D₃ concentration was found to be significantly decreased in iron-deficient rats. We previously demonstrated that a decrease in renal 1 α -hydroxylase activity resulted in decreased serum 1,25-dihydroxyvitamin D₃ concentration, which accounted for decreases bone formation in iron-deficient rats [2]. Although we did not measure renal 1 α -hydroxylase activity in the present study, given our previous findings, it is possible to speculate a reduction in renal 1 α -hydroxylase activity in iron-deficient rats. Additionally, in the present study, serum 1,25-dihydroxyvitamin D₃ concentration in iron-deficient rats was significantly increased following dietary iron supplementation and was similar to that in control rats, although the iron status did not fully recover to the normal range following dietary iron supplementation. Thus, an increase in the serum 1,25-dihydroxyvitamin D₃ concentration due to dietary iron supplementation could increase bone formation in iron-deficient rats. Also, 1,25-dihydroxyvitamin D₃ is responsible for transcriptional regulation of *Bglap* expression [18]. Therefore, an increase in serum 1,25-dihydroxyvitamin D₃ concentration as a result of dietary iron supplementation in the iron-deficient rats could reflect the results of serum osteocalcin concentration and femoral *Bglap* mRNA expression observed in this study. Recently, Qiu *et al.* reported that the mRNA and protein expression of renal CYP27B1 (1α-hydroxylase) were upregulated in iron-deficient rats compared to that in control rats [19]. In the future, the relationship between the mRNA and protein expression of 1α-hydroxylase and its activity should be studied in iron-deficient rats.

Conclusion

We examined the effects of dietary iron supplementation on bone formation markers and serum 1,25-dihydroxyvitamin D₃ concentration in iron-deficient rats. Iron deficiency led to decreased hemoglobin concentration, liver iron, and serum 1,25-dihydroxyvitamin D₃ and serum osteocalcin concentrations. Although dietary iron supplementation increased hemoglobin and liver iron concentrations, the levels were not restored to normal. However, dietary iron supplementation restored serum 1,25-dihydroxyvitamin D₃ and osteocalcin concentrations and femoral mRNA expression of *Bglap* and *Col1a1* in iron-deficient rats. These results suggest that dietary iron supplementation can potentially reverse the effects of iron deficiency on bone health by increasing bone formation.

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Regular article

Acute methylmercury exposure induces inactivation of ATR pathway and enhancement of DNA replication stress through degradation of Rad17

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Abstract

Methylmercury (MeHg) is a neurotoxic chemical of significant public health concern. Acute exposure to MeHg causes inhibition of cellular proliferation and DNA replication in cellular and animal models, and impedes S-phase entry and progression in several types of primary and cultured cells. However, the effect of MeHg on the DNA damage response remains unknown. The ATM and ATR pathways are two major components of the DNA damage response that regulate cell cycle progression. Here we report that acute MeHg exposure enhanced DNA replication stress in S phase. MeHg also inhibited the kinase activity of ATR, suggesting that replication forks collapsed in S phase. Moreover, MeHg promoted the degradation of Rad17 as a possible mechanism underlying the inactivation of ATR. These results suggest a novel connection between acute MeHg exposure and the ATR pathway, providing insight into a yet unidentified mechanism of the inactivation of Rad17 and the ATR pathway.

Keywords : Methylmercury, DNA damage response, DNA replication stress, ATM, ATR, Rad17

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Abbreviations : MeHg, methylmercury; ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3-related; DSB, DNA double-strand break; UV, ultraviolet; 9-1-1 complex, Rad9-Hus1-Rad1 complex

Introduction

ATM- and ATR-dependent checkpoint signaling pathways are two major components of the DNA damage response. The kinase activity of ATM is activated by DNA double-strand breaks (DSBs), leading to the phosphorylation of downstream substrates, including Chk2 and Kap1, to promote DSB repair. Various chemically modified DNA, including DSBs, are converted into single-

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Received: April 26, 2023 Accepted: June 8, 2023 Released online: June 26, 2023 stranded DNA, including DSBs, are converted into singlestranded DNAs. Single-stranded DNA regions in the genome, which are also generated by the inhibition of DNA replication through polymerase-helicase uncoupling, activate the ATR pathway, and the ATR pathway stabilizes arrested replication forks. Thus, the inhibition of ATR leads to the collapse of replication forks, resulting in the conversion of the arrested forks into DSBs. ATR phosphorylates Chk1 and downstream substrates to promote cell cycle arrest [1-3].

Rad17 initiates the activation of the ATR pathway. It recognizes a junction between single-stranded DNA and doublestranded DNA to bind damaged chromatin and interacts with the Rad9-Hus1-Rad1 complex (9-1-1 complex) to load the complex



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onto the damaged chromatin [4-6]. The 9-1-1 complex recruits TopBP1, and this interaction activates the kinase activity of ATR [7, 8]. The activated checkpoint proteins are inactivated by dephosphorylation and proteasomal degradation; Rad17 [9], Chk1 [10], and Claspin [11-13] have been reported to undergo proteasome-dependent protein degradation. We recently showed that the degradation of Rad17 is promoted by the nuclear translocation of Rad17 through the N-terminal D-boxes [14].

Methylmercury (MeHg) is a neurotoxic chemical of significant public health concern according to the World Health Organization [15, 16]. Acute MeHg exposure causes inhibition of cellular proliferation and DNA replication in cellular models [17-19], hinders DNA replication in the developing brain of a rat model [19], and impedes S-phase progression in murine erythroleukemia cells [20]. The inhibition of S-phase entry due to acute MeHg exposure has also been observed in primary cerebellar granule and cortical precursor cells, with a reduction in cyclin E protein levels [19]. The p21 protein, an inhibitor of cyclin-dependent kinases, has been suggested to be involved in cell cycle arrest after MeHg exposure [21]. The toxicity of MeHg is at least partly attributed to the generation of reactive oxygen species [22], and oxidative DNA damage is a major type of DNA damage targeted by the ATM and ATR pathways. Yet, little is known about the effects of acute MeHg exposure on the ATM and ATR pathways. Here we demonstrate that acute MeHg exposure enhances DNA replication stress in S phase.

Materials and Methods

Cell culture and plasmids

COS-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) high glucose (D5796, Sigma-Aldrich) supplemented with 5% bovine serum (16170-078, Gibco) and 1% fetal bovine serum (FB-1290/500, Biosera). Human Rad17 (isoform 1, NCBI NP_579921.1) was cloned in a pcDNA3 vector with an N-terminal flag tag [6].

Antibodies

The following antibodies were used: FLAG (PM020, Medical & Biological Laboratories), Hsc70 (sc7298, Santa Cruz Biotechnology), Chk1 (2360, Cell Signaling Technology), Chk1-pSer345 (2348, Cell Signaling Technology), Chk2-pThr68 (2661, Cell Signaling Technology), Kap1 (A300-274A, Bethyl Laboratories), and Kap1-pSer824 (A300-767A, Bethyl Laboratories).

Effect of MeHg on the ATM pathway

COS-1 cells were seeded at 2×10^5 cells per well in 12-well plates, incubated overnight at 37 °C with 5% CO₂, and treated with 4 mM thymidine for 20 hours. The cells were exposed to 20–40 μ M MeHg (M0589, Tokyo Chemical Industry) or 5 mM caffeine for 5 hours in the presence of thymidine, and SDS-lysate was prepared. Mock and thymidine-exposed samples were prepared in duplicate for each experiment.

Effect of MeHg on Chk1

COS-1 cells were seeded at 4×10^5 cells in 35-mm dishes and incubated overnight. The medium was removed, and the cells were irradiated with 30 J/m² UV-C (256 nm) at 135 μ w/cm². The UV-irradiated cells were exposed to 10–40 μ M MeHg or 5 mM caffeine for 5 hours, and SDS-lysate was prepared. Samples were prepared in singlicate or duplicate for each experiment.

Effect of MeHg on Rad17

COS-1 cells were seeded at $4 \times 10^{\circ}$ cells in 35-mm dishes and incubated overnight. Cells were transfected with 1 µg of pcDNA3/flag-Rad17 or pcDNA4-TO vectors with 5 µL Lipofectamine 2000 (11668-019, Invitrogen). Twenty-four hours after transfection, the cells were divided into four wells of a 24-well plate and incubated overnight. The cells were exposed to 10–40 µM MeHg for 6 hours, and SDS-lysate was prepared. Mock-exposed samples were prepared in duplicate for each experiment.

MTS assay

COS-1 cells were seeded at 2×10^4 cells per well in 96-well plates and cultured overnight. The cells were exposed to 20 or 40 μ M MeHg for 5 hours. The medium was changed to fresh medium, and 20 μ L of CellTiter 96 solution (G3580, Promega) was added to each well. The cells were incubated for 3 hours, and the absorbance at 490 nm was measured using a SpectraMax ABS (Molecular Devices). Cell viability was calculated and presented as mean ± standard deviation.

Results

Acute exposure to MeHg induced caspase-independent cell death in COS-1 cells

We evaluated the effect of acute exposure to MeHg in COS-1 cells, which are derived from the kidney of an African green monkey. This cell line has been immortalized using SV40 large T antigen and is not derived from tumors. The effects of MeHg on cell viability were examined by the MTS assay. The exposure to 20 and 40 µM MeHg decreased cell viability to 63% and 7%, respectively (**Figure 1**). Previous studies have shown that MeHg-induced cell death involves both caspase-dependent and caspase-independent pathways [23, 24]. We examined the effect of caspase inhibitors Z-VAD-FMK and Ac-DEVD-CHO on MeHg-induced cell death. However, these inhibitors did not rescue cell viability (data not shown), suggesting that MeHg-induced cell death is not caspase-dependent under the present experimental conditions.



Figure 1. Cell viability analysis by the MTS assay.

COS-1 cells were exposed to 20 or 40 μ M MeHg for 5 hours, and cell viability was examined by the MTS assay. The graph represents results from six independent experiments. *P* values were calculated using Student's *t*-test.***, *p* < 0.001.



Figure 2. MeHg exposure activated the ATM pathway in the presence of replication stress.

(A) COS-1 cells were exposed to thymidine for 20 hours, followed by exposure to MeHg for 5 hours. SDS-lysate was prepared and probed with the indicated antibodies. Caffeine was used as a positive control.

(B, C) Graphs represent results from more than three independent experiments. *P* values were calculated using a paired *t*-test. *, p < 0.05. **, p < 0.01. ***, p < 0.001.

MeHg exposure activated the ATM pathway in the presence of replication stress

We evaluated the effect of MeHg on the ATM pathway. ATM phosphorylates Kap1-S824 in response to DSBs [25]. Thymidine exposure induces replication stress through the depletion of dCTP [26]. The thymidine-exposed cells showed a significant increase in Kap1 and Chk2 phosphorylation (**Figures 2A–C**), consistent with our previous study [27]. The thymidine-induced increase in Kap1 phosphorylation was further enhanced by caffeine exposure (**Figure 2B, lanes b and c**). Previous studies have reported that caffeine inhibits ATR and ATM *in vitro* but acts as an activator of these kinases *in vivo* [27, 28]. This discrepancy is attributed to the fact that the inhibition of ATR under replication stress results in replication fork collapse to activate ATM (**Figure 5**). MeHg exposure also induced Kap1 and Chk2 phosphorylation (**Figures 2B, C, lanes g and j**). Co-exposure to MeHg and thymidine resulted in significantly higher phosphorylation of Kap1 and Chk2 (**Figures 2B, C, lanes f and i**). In other words, MeHg and thymidine had a synergistic effect on Kap1 and Chk2 phosphorylation (**Figure 2B, lanes f and i**), suggesting that MeHg exposure enhances the kinase activity of ATM. Our anti-Chk2 antibody did not detect the Chk2 protein in COS-1 cells (data not shown). These results raised the possibility that the arrested replication forks due to thymidine exposure were converted into DSBs through replication fork collapse upon MeHg exposure.

MeHg exposure inactivated the ATR-Chk1 pathway

The synergistic effect of MeHg and thymidine raised the possibility that MeHg exposure might have led to the inactivation of the ATR pathway. We examined the effect of MeHg on the ATR pathway following UV irradiation. Chk1-S345 is a well-known ATR-dependent phosphorylation site [1]. As reported previously, UV irradiation induced Chk1 phosphorylation in COS-1 cells, which was suppressed by caffeine exposure (**Figures 3A, B**). These results suggest that the kinase activity of ATR was inhibited by caffeine. Moreover, MeHg exposure decreased Chk1 phosphorylation (**Figures 3C, D**), suggesting that MeHg inhibits ATR-dependent Chk1 phosphorylation and induces replication fork collapse by inhibiting ATR in the presence of replication stress (**Figure 2**).

MeHg exposure promoted degradation of Rad17

We examined the effect of MeHg on the components of the ATR pathway to elucidate the mechanism by which MeHg inactivates ATR-Chk1 signaling. Chk1 has been reported to undergo proteasome-dependent protein degradation [10]; however, MeHg exposure did not decrease the amount of Chk1 (Figure 3C). Rad17 is essential for activating and maintaining the ATR-Chk1 pathway. We examined the amount of Rad17 using an exogenous flag-Rad17 protein, as our anti-Rad17 antibody did



Figure 3. MeHg exposure inactivated the ATR-Chk1 pathway.

COS-1 cells were irradiated with 30 J/m² UV-C, and the cells were recovered for 5 hours in the presence of caffeine (A, B) or MeHg (C, D). SDS-lysate was prepared and probed with the indicated antibodies. Graphs represent results from two (B, n = 3) or three (D) independent experiments. Ratios of Chk1-pS345/Hsc70 are shown (B, D). *P* values were calculated using Student's or Welch's *t*-test. ***, p < 0.001.

not detect endogenous Rad17. Exposure of COS-1 cells to 20 or 40 μM MeHg decreased the amount of Rad17 to 87% and 37%, respectively (**Figure 4**). We also examined the effect of MeHg on Rad17 K/R359–363A mutant that shows resistance to proteasomal degradation [14]. The amount of Rad17 K/R359–363A mutant was also reduced after MeHg exposure (data not shown). In our previous work, flag-Rad17 wild type and K/R359–363A mutant showed a half-life of 7.9 and 34.7 hours, respectively [14]. Given that MeHg exposure reduced the amount of Rad17 within 5 hours, it is likely that the decrease in Rad17 was due to protein degradation and not the inhibition of transcription or translation. These findings suggest that MeHg exposure inactivates the ATR pathway through Rad17 degradation. Moreover, as the exposure to 10 μM MeHg increased the amount of Rad17 (**Figure 4**), MeHg-induced cellular stress may upregulate Rad17 before degradation.

We also examined the effect of Z-VAD-FMK, a pan-caspase inhibitor, on the MeHg-induced Rad17 degradation. However, Z-VAD-FMK did not prevent Rad17 degradation (data not shown). The mechanism underlying the degradation of Rad17 remains to be elucidated.

Discussion

MeHg is classified by the International Agency for Research on Cancer as a Group 2B compound, indicating its potential carcinogenicity in humans. MeHg exposure has been shown to induce DNA strand breaks [29-33], mutations [34], and chromosomal abnormalities [32] in cellular models. The possible mechanisms include direct oxidation of the DNA and inhibition of DNA repair machinery. In this study, we have demonstrated that acute exposure to MeHg inhibits the ATR pathway (**Figure 3**) and also induces DSBs (**Figure 2**). Our findings suggest that that the inhibition of ATR-dependent DNA damage responses may also be a possible mechanism underlying HeMg-induced DNA strand breaks and mutations.

We examined the effect of acute MeHg exposure on the ATR pathway several hours later. Despite a decrease in the MTS score to 7%, the expression of proteins remained unaffected (**Figures 2–4**). The MTS assay monitors mitochondrial activity, and it has been demonstrated that MeHg inhibits mitochondrial function [35]. We speculate that MeHg suppresses mitochondrial function before the onset of cell death. Previous studies have shown that exposure to MeHg can cause chromosome abnormalities in experimental animals [36, 37]. Under physiological conditions, chronic exposure to MeHg may inhibit the ATR pathway, resulting in the accumulation of chromosome abnormalities observed in experimental animals.

Previous studies have reported that the ATR pathway prevents apoptotic responses upon replication stress and UV irradiation [38, 39]. ATR also plays a direct anti-apoptotic role in mitochondria [40]. Our results show that MeHg exposure leads to Rad17 degradation (Figure 4), which inactivates the ATR pathway (Figure 3). In the presence of replication stress, the ATR pathway stabilizes stalled replication forks. However, the inactivation of ATR can cause stalled replication forks to collapse, leading to the formation of DSBs, which are the most cytotoxic type of DNA lesion [1, 2] (Figure 5). Because exposure to MeHg caused replication stress and DSBs by deactivating ATR (Figures 2, 3), the inactivation of ATR and the presence of DSBs contributed to cell death, in addition to other cytotoxic effects of MeHg.



Figure 4. MeHg exposure promoted Rad17 degradation.

- (A) COS-1 cells were transfected with flag-Rad17. Forty-eight hours after transfection, the cells were exposed to 10–40 μM MeHg for 6 hours. SDS-lysate was prepared and probed with the indicated antibodies. All lanes were obtained from the same blot.
- (B) The graph represents results from more than three independent experiments. Rad17 protein amount is shown as the ratio of FLAG/Hsc70. *P* values were calculated using Student's *t*-test. *, *p* < 0.05. ***, *p* < 0.001.



Figure 5. | MeHg exposure inactivates the ATR pathway, leading to the induction of DSBs.

Thymidine exposure, which induces replication stress, and UV irradiation activate the ATR pathway, leading to the stabilization of stalled replication forks. If the ATR pathway is inactivated, the stalled replication forks collapse and are converted into DSBs, which activates the ATM pathway. In the diagram depicted, exposure to MeHg degrades Rad17, which in turn prevents ATR activation.

Rad17 is an important activator and regulator of the ATR pathway. Our results revealed a novel connection between acute MeHg exposure and the ATR pathway. Cell cycle progression resumes after DNA damage repair, which is not a spontaneous but an actively regulated process that involves the inactivation of DNA damage checkpoints. Our findings also suggest the presence of an unidentified mechanism of Rad17 degradation leading to the inactivation of the ATR pathway.

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CRediT Author Statement

Y. F.: conceptualization, methodology, formal analysis, investigation, resources, writing—original draft, visualization, project administration, funding acquisition. D. W.: formal analysis, investigation, visualization. Y. T. and N. S.: resources, supervision, funding acquisition. Y. O.: resources, writing—review and editing, supervision, project administration, funding acquisition.

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Regular article

Regional characteristics of 40 elements in water around Chornobyl nuclear power plant after some decades since the accident

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Abstract

Concentrations of 40 kinds of elements in water samples collected around Chornobyl nuclear power plant (CNPP) in September 2017 were determined using ICP-MS to investigate possible adverse effects on residents' health. Regional characteristics of elements of Chornobyl and Narodychi district, which suffered high radioactivity due to the accident on 1986, were compared with those of other cities in Ukraine, Belarus, and Japan. The concentrations of toxic elements such as As, Cd, Pb, and Se in Chornobyl and Narodychi were lower than regulation values of WHO guidelines for drinking-water quality, and not particularly higher than those of other cities. Among three well water samples of Chornobyl, one or two contained much higher concentrations of Mn, Zn or La than tap water or hand washing water. Three well water and one tap water samples of Chornobyl were also collected in April 2018, August 2019, and January 2020 in order to know the yearly change of elements. Changes in concentrations of most of samples were lower than 0.3 μ g/L, but an extremely high value of 340 μ g/L was observed temporarily in one well water sample in 2019, although the source of Pb was not clear. These results did not suggest notable health problems associated with elements in the water of Chornobyl.

Keywords: Drinking water quality, Metal elements, Chornobyl, ICP-MS

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Introduction

No one can live without water. Adults need about 2.5 liters of water a day, of which two liters are taken as drinks such as simple water, tea, coffee, juice, and so on. Most people consume simple water, for example, tap water, well water, or bottled water. The developed countries have water supply systems and serve hygienic drinking water to residents. Even in the developed countries, however, municipal water pipes do not reach secluded places in



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the mountain areas. In developing countries, there are many cases in which rural areas do not always have water supply systems. The residents living around these areas use nearby water such as well water, surface (river or pond) water or rain water. These waters are not always tested regarding security for health. Natural water contains various minerals coming from environmental conditions or chemical contaminants caused by agricultural or industrial residues. WHO has established criteria of drinking water in order to prevent negative effects of human health caused by drinking water [1]. In our research, we have been paying attention to water quality in terms of its elements. We collected water samples and analyzed various elements, then considered their health effects. Previously, we reported the results of water samples collected in the People's Republic of Bangladesh with arsenic contamination from groundwater, and the Republic of Kazakhstan with environmental problems from the Aral Sea [2].

In the present study we paid attention to Ukraine and Belarus, which suffered environmental destruction from radioactive materials generated by explosions of the Chornobyl Nuclear Power Plant (CNPP) about 30 years ago. We visited Ukraine and Belarus in September, 2017. We obtained permission to get into the 30 km area of the CNPP in advance. In Ukraine we collected water samples in the CNPP area and in the Narodychi Raion (district) of Zhytomyr Oblast, located 50 - 70 km south-west from CNPP, known for its high radioactivity due to the accident of 1986, and also in the capital, Kyiv. The southern Belarus area was heavily exposed to radioactivity by the accident of CNPP. We also collected water samples in the capital, Minsk, and Brest, in south-west Belarus. For comparison, tap water and bottled water of Japan were also analyzed. We went to Ukraine again in April, 2018, August, 2019, and January, 2020, and collected well water that was used for daily life by residents within 10 - 30 km of CNPP and tap water of a guest house in Orane village of Chornobyl. We focused on regional characteristics of element concentrations of water around CNPP. Concentrations of 40 kinds of elements in water were determined and we discuss their effects on residents' health,

Materials and Methods

Water samples

Water samples were collected in polypropylene containers which were pre-cleaned by soaking in 35% nitric acid solution for 2 days and rinsed with ultrapure water ($18.2 \text{ M}\Omega/\text{cm}$).

The water samples of 2017 (n=37) were divided into 5 groups according to the water type. a) Mineral water; bottled drinking water with trademark of "mineral water". b) Drinking water; water in containers provided for drinking in commercial products. c) Tap water; supplied by the local governments; users use it as drinking water. d) Well water: usual well water without any specialized treatment. e) Hand-washing water; piped water supplied at toilets or wash rooms for hand washing. The numbers and regions of samples are listed in **Table 1**.

Methods

Water samples were passed through 0.45 µm filter membranes, and then diluted 5 times with 0.5% nitric acid prepared with 68% HNO₃ (ultrapure grade; Tama Chemicals Co., Kawasaki, Japan) and ultrapure water. Twenty µg/L Tb was added as an internal standard. The elemental concentrations in each diluted

Table 1.	Water	sample	of 2017	(n=37)
	vvucci	Jumpic	012017	(11-57)

Region	Group	Notation in Figures	Number
	Drinking water	Drinking	1
	Tap water	Тар	1
Chernovyl	Well water	Well	3
	Hand-washing water	Hand	2
Narodychi district	Tap water	Тар	1
	Mineral water	Mineral	2
Kyiv	Drinking water	Drinking	1
	Hand-washing water	Hand	3
	Mineral water	Mineral	1
	Drinking water	Drinking	2
Minsk	Tap water	Тар	1
	Hand-washing water	Hand	9
	Mineral water	Mineral	1
Brest	Drinking water	Drinking	1
	Hand-washing water	Hand	6
2T 1	Drinking water	Drinking	1
Iokyo	Tap water	Тар	1

solution were determined using an inductively coupled plasma mass spectrometer (ICP-MS; Agilent 8800; Agilent Technologies, Tokyo, Japan). As standard solutions, 0, 0.4, 4.0, and 40.0 μ g/L of the multi-element standard solutions XSTC-1 and XSTC-13 (SPEX Industries Inc., NJ, USA) diluted with 0.5% nitric acid were used. The concentration of 40 elements was calculated using standard calibration curves with the signal intensity of Tb as the internal standard. Certified reference materials (JSAC-0301-3b, JSAC-0302-3c, Japan Society for Analytical Chemistry) were analyzed for assessing the validity of measurements.

Operation conditions of ICP-MS were as follows. RF power: 1550 W, RF matching 1.50V, sampling position: 10 mm, plasma gas flow: 15 L/min. carrier gas flow: 1.03 L/min. For 2017 samples, ⁷Li, ⁹Be, ²⁷Al, ³⁹K, ⁵¹V, ⁸⁵Rb, ⁹⁰Zr, ¹¹¹Cd, ¹¹⁵In, ¹²³Sb, ¹³³Cs, ¹³⁵Ba, ¹³⁹La, and ¹⁹³Ir were measured by no gas mode, and ²³Na, ⁵²Cr, ⁵⁵Mn, ⁵⁹Co, ⁶⁰Ni, ⁶³Cu, ⁶⁶Zn, ⁷⁴Ge, ⁷⁵As, ⁷⁸Se, ⁸⁸Sr, ⁹⁸Mo, ¹⁰³Rh, ¹⁰⁷Ag, ¹¹⁸Sn, ²⁰⁵Tl, ²⁰⁸Pb, ²⁰⁹Bi, ²³²Th, and ²³⁸U were measured using He as collision gas at 5mL/min. ⁵⁶Fe was measured using H₂, and ⁴⁰Mg, ⁴⁴Si, ⁴⁷P, ³²S, and ⁶⁰Ca were measured using O₂ as reaction gas at 7 and 0.3 mL/min, respectively. These samples were also measured by no gas mode, and the results were compared with those obtained by collision or reaction gas mode. The concentrations measured by different mode coincided with each other although the sensitivities to several elements by no gas mode were lower, indicating the differences in analysis mode did not affect the results. Measurement of samples collected in 2018, 2019, and 2020 were performed by no gas mode.

Instrument detection limits were defined as the concentrations 3-times the standard deviations (n=10 times measurements) of signal intensities of 0.5% nitric acid. The water hardness was defined as the amounts of CaCO₃ (mg/L), which was calculated from amounts of Ca and Mg obtained by ICP-MS. Molecular weight of CaCO₃ is 100.1, atomic weights of Ca and Mg are 40.1 and 24.3, respectively. The hardness is, therefore, calculated as Ca (mg/L) × 2.5 + Mg (mg/L) × 4.1.

Results

Analytical results of element concentrations of water samples of 2017 are listed by region in **Table 2**. Results of nine elements and one index of individual samples are indicated in **Figures 1A -1J**.

1. Group 1 elements (alkali metals)

Among alkali metals (Li, Na, K, Rb and Cs) in **Table 2A**, the concentration of Li was $0.06 - 25.13 \mu g/L$, where the average values of Chornobyl and Narodychi district were a little higher than those in other areas. Average concentrations of Na showed similar area dependencies to Li, and individual differences of samples were significant as shown in **Figure 1A**. The highest concentration of 41 mg/L was found in the mineral water bought from Minsk railroad station, the second highest was drinking water from Chornobyl, and the third highest was the tap water from Narodychi district. Average concentration of K in Chornobyl samples was higher than the other areas. Among Chornobyl samples, two kinds of well water showed especially high levels of K (**Figure 1B**). Rb concentrations were low; several $\mu g/L$ or lower. Cs was scarcely detected in all samples.

2. Group 2 elements (Alkaline earth metals)

Of the alkaline earth metals, concentrations of Be were lower than detection limit ($0.07 \mu g/L$) except for one well water C of 0.08 $\mu g/L$. The highest concentration of Mg was observed in Narodychi district, and average concentration of Minsk was followed by the other four regions. The highest concentration of Ca was also observed in Narodychi district, which was about 2 or 3 times the average concentrations in Chornobyl, Kyiv, Minsk, and Brest, and about 4 times that of Tokyo. The concentrations of Mg and Ca of individual specimens, and hardness calculated as CaCO₃ are shown in **Figure 1C**, **1D** and **1E**. Concentrations of Sr in Chornobyl and Narodychi district were a little higher than in Kyiv and Brest, and much higher than in Minsk and Tokyo. Concentrations of Ba were less than 120 $\mu g/L$, except for one sample of mineral water from Minsk of 773 $\mu g/L$.

3. Group 3-12 elements (transition elements)

The results of transition elements are shown in **Table 2B**. Zirconium (group 4) concentrations were lower than 0.2 μ g/L, except for well water B in Chornobyl (1.70 μ g/L). Average concentration of V (group 5) was more than 1.0 μ g/L in Chornobyl and Tokyo, but was less than 0.25 μ g/L in other regions. Average concentrations of total Cr (group 6) in each area were from 0.08 to 0.37 μ g/L, and the maximal value was 1.12 μ g/L in well water B of Chornobyl, which was much lower than the 50 μ g/L of the WHO guideline value [1]. Molybdenum (group 6) concentrations were lower than 1.14 μ g/L. Health-based guideline

value of water has not been proposed for Mo.

Average concentration of Mn (group 7) of Chornobyl, 33 μ g/L, was higher than that of other regions (**Table 2B**). As shown in **Figure 1F**, Mn concentrations of two samples, hand-washing B of Chornobyl and tap water of Narodychi district, were higher than the 80 μ g/L of the WHO guideline [1]. Mn concentrations in other samples were lower than 10 μ g/L, except for well water A in Chornobyl (44.2 μ g/L), hand-washing waters D, L, and R in Kyiv, Minsk, and Brest (21.6, 10.8, and 13.8 μ g/L). Iron (group 8) concentrations were lower than 2 μ g/L, except for one well water of 53 μ g/L which was much lower than that in Japan and that of the USEPA criterion of 300 μ g/L [3, 4]. Cobalt (group 9) concentrations were lower than 0.8 μ g/L. Rhodium (group 9) was detected in several samples at 2.9-12.8 μ g/L. Iridium (group 9) was under detection limit in all samples. Nickel (group 10) concentration was 10.76 μ g/L in Narodychi district and average concentration was 2.13 μ g/L in Chornobyl. These values were higher than in other regions, but were much lower than the 70 μ g/L of the WHO guideline [1].

Copper (group 11) was found in almost all samples, and average concentrations of each region were from 3 to 33 μ g/L. The highest concentration of 125 μ g/L was much lower than the 2000 μ g/L of the WHO criterion [1], 1000 μ g/L of Japan [3], and 1300 μ g/L of USEPA [4]. Silver (group 11) concentrations were lower than 0.120 μ g/L, except for the 2.69 μ g/L of drinking water in Kyiv. The concentrations of Zn (12 group) of Chornobyl and Narodychi district were higher than those in the other regions. As shown in **Figure 1G**, the concentrations of Zn in most samples were lower than 400 μ g/L, except for the 4830 μ g/L of well water A of Chornobyl , 2122 μ g/L of hand-washing water L of Minsk, 1382 μ g/L of hand-washing S of Brest and 948 μ g/L of Narodychi district. Cd (group 12) concentrations were lower than 0.24 μ g/L in the tap water of Narodychi district, which was much lower than the WHO criterion of 3 μ g/L [1].

4. Group 13-16 (base metal and nonmetal)

Analytical results of base metal (Group 13 -15) and nonmetal (Group 14-16) are shown in **Table 2C**. Aluminum (group 13) concentrations were very low in Narodychi district, Brest and Tokyo, 3 μ g/L or lower, and varied from under detection limit to 150 μ g/L in Chornobyl, Kyiv and Minsk. Indium (group 13) and Tl (group 13) concentrations were scarcely detected or under detection limit in all samples. Germanium (group 14) was detected in most samples, however, the concentrations were very low, less than 0.2 μ g/L. Tin (group 14) concentrations were under detection limit except in 2 samples. Lead (group 14) was detected in many samples but the concentrations were low, as shown in **Figure 1H**. There were only 2 samples with the concentration more than 1 μ g/L, and the highest concentration of 1.47 μ g/L in the hand washing water I of Minsk was much smaller than the WHO criteria of 10 μ g/L [1]. Antimony (group 15) concentrations were lower than 1.4 μ g/L (guideline value is 20 μ g/L). Bismuth (Group 15) concentrations of the samples were very low, 0.05 μ g/L or less.

Silicon (group 14), one of nonmetal elements existing abundantly in the Earth's crust, was detected in all samples from 108 to 14,863 μ g/L. The concentrations of P (group 15) were lower than 100 μ g/L, except for 650 μ g/L of well water C. Arsenic (group 15) concentrations were from under detection limit (<0.003) to 1.82 μ g/L and were much lower than the 10 μ g/L of the WHO criterion [1]. Sulfur (group 16) concentrations were lower than 300 μ g/L, except for about 1,200 μ g/L in tap water of Narodychi district. Selenium (group 16) concentrations of all samples were lower than 0.8 μ g/L, much lower than the 40 μ g/L of the WHO criterion [1].

5. Lanthanoid and actinoid elements

The results of lanthanoid and actinoid elements are shown in **Table 2C**. The average concentration of La was high in Chornobyl. As shown in **Figure 1I**, La was detected in well water B and C at about 18,000 μ g/L, and in the well water A at 693 μ g/L. La concentrations of all other samples were lower than 130 μ g/L. Among actinoid elements, Th was scarcely detected in all samples, however, U was detected, and the concentrations were different depending on the area. As shown in **Figure 1J**, U concentrations of Chornobyl, Narodychi district, Brest and Tokyo were very low, from under detection limit to 0.12 μ g/L, and most samples of Minsk and two samples of Kyiv were from 0.2 to 1.5 μ g/L.

6. Yearly changes in elemental concentrations

We observed yearly changes in element concentrations of three well waters and tap water of Chornobyl from 2017 to 2020. As shown in **Figure 2**, changes in concentrations of alkali and alkaline earth elements were relatively small, and those of transition

elements were large. Concentrations of Mn in well water A changed from under detection limit to 126 µg/L, and those of Zn from 69 to 3240 µg/L. Manganese in well water B and C also changed from under detection limit to 0.82 µg/L, Zn in well water B and C changed from 15 to 85 µg/L.

Lead concentrations of most samples were lower than 0.3 μ g/L, however, an extremely high value of 340 μ g/L was observed in well water A of 2019. Lead concentration of the next year, 2020, was reduced to 0.3 μ g/L again.

Discussion

The results of determinations of elements in water samples collected in 2017 suggested that the water of Chornobyl and Narodychi district did not contain alarming levels of pollution of toxic elements, compared with other cities of Ukraine, Belarus and Tokyo; not only for drinking water but also for hand-washing water. Elemental characteristics of mineral water sometimes showed quite different patterns from other types of water from the same region, suggesting differences in the original water source. For drinking water, purification or disinfection methods, or materials of containers are thought to affect the element contents.

Among alkali metals, a maximum value of Na about 40 mg/L was observed in mineral water bought at Minsk railroad station. A health-based guideline value for Na has not been derived by WHO [1], and this value was much lower than the 200 mg/L (200,000 μ g/L) of the average taste threshold for sodium, which is within the water quality standards of tap water in Japan [3] for usability in terms of taste. Potassium concentrations of well water A and C, 36 and 43 mg/L, were much higher than those of other samples. Drinking water quality standard of K has not been fixed by WHO [1]. Potassium is an essential element for humans, however the estimated amounts from drinking water were very small and were not of health concern.

Cesium was measured as a stable isotope ¹³³Cs by ICP-MS, and was scarcely detected in all samples. Radioactive Cesium ¹³⁴Cs and ¹³⁷Cs were released by the accident [5]; however, we do not discuss short-lived ¹³⁴Cs (2 years of half-life) here because there are no radiological concerns after more than 30 years. The possibility of the existence of a detectable level of radioactive ¹³⁷Cs is thought to be extremely low because the counts at m/z 137 matched the expected counts of ¹³⁷Ba estimated from ¹³⁵Ba. For the determination of ¹³⁷Cs by ICP-MS, chemical separation of isobaric interference such as ¹³⁷Ba and pre-concentration will be needed [6]. The long-lived (30 years of half-life) ¹³⁷Cs and ⁹⁰Sr have been major components of the aquatic ecosystems except during the first several months after the accident in 1986 [5]. Behavior of released radionuclides has been investigated for a long time [5, 7, 8]. The low risk of consumption of ¹³⁷Cs via the drinking water pathway, in comparison with external and internal (foodstuff) radiation, has been suggested [5,7]. A recent report on 35 years of radiological monitoring of groundwater in the Chernobyl Exclusion Zone indicated that ¹³⁷Cs and ⁹⁰Sr concentrations of well water samples taken in 2019 used by residents in the 10 – 30 km zone were less than the WHO drinking water standard, 10 Bq/L [8]. We reported that the tap water of Semipalatinsk, which was contaminated with radionuclides in the past, did not contain toxic elements presenting health problems [2]. Semipalatinsk in East Kazakhstan, is known as the site of 456 nuclear tests of the Soviet Union's nuclear weapons, conducted from 1949 until 1989. The site was closed in 1991.

Of the alkaline earth metals, Ca, Mg and water hardness calculated as CaCO₃ of individual specimens indicated that the three well waters of Chornobyl showed lower values compared with tap and hand-washing water in this area, suggesting differences between components of surface water and ground water. Three well water samples, mineral water B, drinking water B and C, and Japanese water showed low hardness of 100 or less, but most water in Ukraine and Belarus showed hardness of from 120 to 400. The black fertile soil of Ukraine is known to contain rich humus which retain water and nutrients such as Ca and Mg. Health-based guideline value is not proposed for hardness in drinking-water.

WHO criterion has not been indicated for Zn (12 group) because of no health disorder dangers [1]. Drinking water quality standards of Japan [3] and USEPA [4], however, are 1000 and 5000 µg/L, respectively, for acceptability of drinking water regarding color and taste. Figure 1G suggested that well water A in Chornobyl of 2017 contained about 5000 µg/L Zn and that it is not so tasty for drink, but no problem for health.

Aluminium is abundantly present in the Earth's crust or derived from flocculant during water purification process. WHO criterion has not been indicated, but drinking water quality standard of Japan (200 μ g/L) [3] and USEPS (50-200 μ g/L) [4] are indicated for reason of coloring. Some samples in Chornobyl, Kyiv and Minsk exceed 50 μ g/L.

Cd (group 12), Pb (group 14), As (group 15) and Se (group 16) concentrations of 2017 samples were much lower than those in of the health-based guideline values of WHO criteria, suggesting elemental safety. The result of La (**Figure 1I**) suggests that

La exists in the ore or soil of this area and that it is partially dissolved in ground water. The provisional guideline value of U concentration of WHO is 30 μ g/L [1], considering chemical toxicity. The average concentration of U in Minsk, 0.53 μ g/L, and the highest value of 1.55 μ g/L were much lower than guideline value and were not a health problem. We reported that U concentrations of piped, shallow well, and river water from near Aral Sea region of Kazakhstan were from 18 to 20 μ g/L, but those of deep well were 0.27 μ g/L [2]. The source of U in Kazakhstan has been thought to be the uranium mines in central Asian countries.

Three kinds of well water of the Chornobyl exclusion zone (CEZ) area have been used daily for drinking and cooking by the inhabitants living in this area by their own will. The results of testing well water showed some element features different from the other samples of the same area; lower concentrations of Mg, Ca, and Sr. In addition, one or two of three well waters contained higher concentrations of K, Mn, Zn, and La than the other sorts of samples. The shallow well water is groundwater from the top of hard bedrock and is thought to be easily affected by the surrounding environment. Elemental composition of drinking well water in the Transcarpatian region in west Ukraine indicated high Fe contents ($53 - 411 \mu g/L$) as common characteristics of the surface and underground water of this area [9]. Elemental composition in underground and surface water is affected by geological features and industrial chemicals. Monitoring data of trace elements in tap water of Kryvyi Rih city in the south of Ukraine, which has an industrial complex, indicated seasonal change of Mn, Cu, and Pb contents [10]. Sampling seasons of the water samples of the present study differed depending on the year, and seasonal changes in rainfall, floods and accompanying sediment movement are thought to affect elements in surface and groundwater. Element concentrations and their yearly or seasonal changes are thought to be different in each well.

Extremely high concentration of Pb of 340 μ g/L in well water A was observed in 2019, and the source of Pb and the reason for the increase were unknown; however, this contamination was thought to have been a temporal phenomenon because Pb concentration of the next year, 2020, was reduced to 0.3 μ g/L again. In well water A, yearly changes in concentrations of Mn and Zn were also large. In the present results, big problems regarding toxic elements were not observed, except Pb in 2019; however, continuous observation is preferable for health protection of inhabitants.

Conclusion

The concentrations of 40 elements in water are shown in this paper. The water samples were collected around the CNPP in Ukraine, Brest, south-west Belarus, Minsk, the capital of Republic of Belarus and Tokyo, Japan. As is well known, the CNPP caused a tremendously large-scale disaster, although it happened more than 30 years ago. There have been various treatments for the water supply. (We did not address this matter.) Various reports on CNPP concerning this accident have been made elsewhere; therefore, we do not address this matter in this paper.

Our objective water samples were ones which general persons nearby can obtain. Our results propose no special indications. Unfortunately, Ukraine and Russia were plunged into war in February 2022. The situation in Ukraine must have changed greatly. This situation may be beyond our imagination. Our water specimens were collected before the outbreak of the war. We expect that our data will be useful in the near future in providing data on common water conditions in a peace time situation in Ukraine.

Author contributions

Shinohara: Organizing data and writing draft, Matsukawa: Analysis and data curation, Kimura: Visit Ukraine and Belarus, sample collection, Yokoyama: Supervision, Chiba: Visit Ukraine and Belarus, sample collection, advise on draft contents.

			<0.07				<0.07				< 0.07				< 0.07	< 0.07	0.08	< 0.07	0.02	0.04			
	6579	5271	5925	16648	3321	4481	6146	19996	1594	4136	11276	9915	3603	3002	6588	17947	14960	1448	4620	6474			
	30199	26146	28173	95024	23097	21827	74639	29262	7 6901	22612	56723	78617	21713	25438	52063	127623	83667	17570	25217	41869			
	131.51	95.08	113.29	282.57	8.43	88.97	213.30	286.46	5/.61	62.70	95.75	398.50	66.99	141.26	245.88	306.66	957.95	45.34	363.82	380.27			
	4.05	4.02	4.03	80.21	0.48	24.75	58.46	7/3.01	14.0/	201.52	107.67	 19.26	9.69	3.61	14.68	117.08	34.80	0.86	13.97	17.43			
	102	87	95	254	126	38	212	261	55	73	188	 237	69	75	157	393	271	53	81	131	300	006	300
М	etallor	nics	Res	earch	202	23; 3	(2) #	#MR2	202	303				re	g35								

4.88 1.13 3.71 6.34

7076 2006 3083 8500

2621 1661 897 4874

1.598 1.248 0.093 3.280

0.006 0.003 <0.01 0.012

Table 2.	Element concentrations in	n water samples co	ollected in Ukraine,	Belarus and Japan	in Sep. 2017
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2.64 3.26 0.36 13.02

8198 10436 1995 41424

2934 3715 638 14942

0.752 1.067 0.093 4.141

0.006 0.004 <0.01 0.019

2.62 1.45

8866 7281

2760 1335

4.701

0.06 4.59

2195 20777

<210 4499

1.940 <0.06 6.052

0.014 0.005 <0.01 0.021

2.87 2.59 3.15

7378 7062 7694

> 2656 2552

0.140 0.985

0.012 <0.01 0.018

0.563

2759

Li

Na

Alkali metals K

0.05

8.6

210

0.06

0.01

0.07

_

Sr 0.03

Ba 0.03

hardness

Rb

Cs

Ве

Mg 2.0

Alkaline earth metals Ca

9.76 9.04 0.28 25.13

11742 10198 1139 30721

17279 15281 4395 43057

3.700 1.922 1.419 6.878

0.008 0.004 <0.01 0.016 200,000

12.77

26627

6642

3.150

0.031

Table 2A

 $(\mu g/L)$

Tadie 20							H	1					
	Element	Zr	V	Cr	Mo	Mn	Fe	Co	N.	Cu	Ag	Zn	
	Detection limit	0.02	0.02	0.07	0.08	0.46	11	0.07	0.02	0.01	0.01	0.7	
	Drinking-water quality standard												
;	WHO			50		80			70	2,000			
Region	USEPA			100		50	300			1,300		5,000	
	Japan			20^{*1}		50	300			1,000		5,000	
		0000	1 22	0 27	000	10 22	C1	016	2 I 2	4 7 1	000	707 5	
(n=7)	SU 0	0 63	1 79	0 47	0 1 8	89 69	18	0 77	2 29	35 2	0 01	18181	
	min	< 0.02	< 0.02	< 0.07	<0.08	0.23	<11	<0.07	<0.02	< 0.01	< 0.01	<0.7	
	max	1.70	4.26	1.12	0.51	186.85	53	0.76	6.54	8.20	0.05	4830.2	
Narodychi Raiot (n=1)		0.02	< 0.02	0.11	0.33	111.74	<11	0.20	10.76	33.05	0.02	948.2	
Kyiv (n-6)	Average	0.03	0.25	0.08	0.32	3.84 8.68	<11	0.12	0.59	1.40 2 35	0.49 1 08	11.4 15.0	
	min	< 0.02	< 0.02	< 0.07	0.09	< 0.46		< 0.07	<0.02	0.01	< 0.01	<0.7	
	max	0.06	0.73	0.20	0.66	21.56		0.22	1.07	5.81	2.69	40.5	
Minsk	Average	0.05	0.26	0.20	0.57	2.17	<11	0.05	0.37	8.10	0.01	239.1	
(n=13)	SD	0.03	0.27	0.27	0.38	3.43		0.03	0.33	11.83	0.00	567.6	
	max	0.09	0.87	0.90	50.00 1.14	<0.40 10.78		0.10	1.10	45.50	0.01	3.7 2121.9	
Brest	Average	0.06	<0.02	0.09	0.06	2.85	<11	0.08	0.69	28.61	0.01	302.0	
(n=8)	SD	0.02		0.08	0.03	4.57		0.03	1.35	44.68	0.00	458.0	
	min	< 0.02		<0.07	< 0.08	<0.46		<0.07	0.07	< 0.01	0.01	< 0.7	
	max	0.07		0.27	0.11	13.81		0.12	4.02	125.45	0.01	1382.4	
Tokyo	Average	0.02	1.24	0.24	0.32	0.79	<11	<0.07	0.03	3.07	0.01	<0.7	
(n=2)	min	< 0.02	1.21	<0.07	0.22	<0.46			< 0.02	0.13	0.01		
	max	0.02	1.28	0.43	0.41	1.35			0.05	6.01	0.01		

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Table 2C Region Chernovyl	Element Detection limit Drinking-water quality standard WHO USEPA Japan Japan	Al 2 I 50-200 200 24	Ge 0.016 0.049	Ise metal Pb 0.05 10 15 10 10 0.07	Sb 0.01 6 0.291	Bi 0.03	Si 22 9370	P 20	Nonmetal As 0.003 10 10 10 10 0.275	S 10	Se 0.004 40 50 10 0.161	Lanthai La 10 5232	<0.
Chernovyl (n=7)	Average SD min max	24 54 <2 146	0.049 0.036 <0.016 0.093	0.07 0.07 <0.05 0.22	0.291 0.502 0.005 1.390	0.06 0.08 <0.03 0.22	9370 2351 6244 13309	101 243 <20 651	0.275 0.438 <0.003 1.176	99 150 <10 331	0.1 <i>6</i> 1 0.278 0.002 0.715	1	5232 8701 <10 8647
Narodychi Raion (n=1)		<2	< 0.016	< 0.05	0.090	< 0.03	10926	<20	0.151	1194	<0.004		<10
Kyiv (n=6)	Average SD min max	47 72 <2 153	0.023 0.018 <0.016 0.048	0.11 0.20 <0.05 0.53	0.110 0.106 <0.01 0.262	0.10 0.21 <0.03 0.53	7546 2051 4860 9843	44 34 93	0.638 0.882 0.014 1.822	20 37 <10 97	0.148 0.180 <0.004 0.373		22 15 <10 42
Minsk (n=13)	Average SD min max	20 27 ~2 75	0.024 0.020 <0.016 0.077	0.28 0.39 <0.05 1.47	0.044 0.054 0.014 0.215	0.28 0.39 <0.03 1.47	3325 1471 629 5109	17 16 <20 59	0.416 0.294 0.015 1.029	31 46 <10 162	0.067 0.168 <0.004 0.581	L ^	26 35 (10
Brest (n=8)	Average SD min max	· ^ 2 · 2	0.046 0.031 <0.016 0.099	0.21 0.44 <0.05 1.28	0.099 0.191 0.014 0.569	0.20 0.44 <0.03 1.28	4968 1995 108 6236	<20	0.041 0.038 0.002 0.100	<10	0.046 0.090 <0.004 0.248		15 8 <10 30
Tokyo (n=2)	Average min max	<2 3	0.117 <0.016 0.195	0.08 <0.05 0.14	0.083 0.045 0.120	0.08 <0.03 0.14	14612 14361 14863	<20	1.031 0.529 1.533	<10 <10 15	0.282 <0.004 0.561		9 <10 14
												1	

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Figure 1. Element concentration in each water sample. (A)Na, (B)K, (C)Mg, (D)Ca, (E)hardness, (F)Mn, (G)Zn, (H)Pb, (I)La, and (J)U.





Figure 2.Time dependent changes in element concentrations of three well
waters and tap water of Chornobyl (2017-2020)

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Note

Changes in bone metabolism-related mRNA expression in irondeficient rats

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Abstract

To determine the underlying mechanism of iron deficiency-induced bone loss, we investigated the effects of dietary iron deficiency on bone metabolism-related mRNA expression in rats. Weanling male Wistar rats were divided into three groups, each comprising six rats. Rats in two of the three groups were given free access to either a control (C group) or an iron-deficient (D group) diet for 4 weeks. Rats in the PF group were pair-fed the control diet with rats in the D group. Hemoglobin concentration, serum osteocalcin concentration, femoral bone mineral content and density, and femoral *Igf1, Bmp2, Runx2, Sp7, Spp1, Bglap*, and *Col1a1* mRNA expression were significantly lower in the D group than in the C and PF groups. Additionally, urinary deoxypyridinoline excretion and femoral *Acp5* mRNA expression were significantly lower in the D group than in the C and PF group than in the C and PF groups. These results suggested that dietary iron deficiency reduced osteoblastogenesis by decreasing the expression of insulin-like growth factor-1 and bone morphogenetic protein 2. Furthermore, bone resorption parameters indicated a decrease in bone resorption in iron-deficient rats.

Keywords: dietary iron deficiency, insulin-like growth factor-1, bone morphogenetic protein 2, bone formation, bone resorption

Statements about COI: The authors declare no conflict of interest associated with this manuscript.

Introduction

Osteoporosis is a bone disorder affected by many factors, such as sex, age, and the environment. Nutritional status has been identified as a risk factor for osteoporosis, and a deficiency or proportional change in calcium, phosphorus, and magnesium

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Received: June 13, 2023 Accepted: July 13, 2023 Released online: July 20, 2023 or proportional change in calcium, phosphorus, and magnesium may lead to bone loss [1]. In addition, iron deficiency induces bone loss, and dietary iron deficiency decreases bone mineral content (BMC) and bone mineral density (BMD) in rats [2-4]. In postmenopausal women, dietary iron intake is associated with BMD [5, 6].

An imbalance in bone turnover between osteoblastic bone formation and osteoclastic bone resorption results in bone loss. The concentrations of bone formation markers, such as serum osteocalcin and procollagen type I N-terminal propeptide (P1NP) concentrations, are decreased in iron-deficient rats [2-4, 7]. Additionally, bone histomorphometric parameters have shown that dietary iron deficiency decreases the bone formation rate and osteoblast surface in rats [3, 4]. Therefore, decreased



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bone formation is thought to cause dietary iron deficiency-induced bone loss. Runt-related transcription factor 2 (Runx2) and Osterix (also known as Sp7) are essential transcription factors for osteoblast differentiation [8, 9]. Dietary iron deficiency decreases femoral Runx2 and Sp7 mRNA expression, suggesting that iron deficiency-induced downregulation of Runx2 and Osterix might influence osteoblastic bone formation [4]. However, the mechanisms by which Runx2 and Osterix are regulated in iron-deficient rats remain unclear.

Díaz-Castro *et al.* demonstrated that dietary iron deficiency increases the serum C-terminal telopeptide of type I collagen (CTx) and tartrate-resistant acid phosphatase (TRAP) concentrations in rats [7]. Consistently, urinary CTx excretion was increased in iron-deficient rats [2, 3]. These results indicated that dietary iron deficiency increases bone resorption. However, dietary iron deficiency decreases urinary deoxypyridinoline (DPD) excretion and reduces osteoclast numbers in rats [3, 4]. The reason for this discrepancy remains unclear.

This study aimed to identify the factors through which dietary iron deficiency inhibits osteoblast differentiation and reduces bone formation. Therefore, we investigated the effects of dietary iron deficiency on bone formation-related mRNA expression in rats. Furthermore, to determine whether dietary iron deficiency increases or decreases bone resorption, we measured bone resorption-related mRNA expression in iron-deficient rats.

Materials and Methods

Ethical approval

This study was approved by the Animal Use Committee of Tokyo University of Agriculture (approval number 280069) and performed in accordance with the university's guidelines for the care and use of laboratory animals.

Experimental design

Eighteen 3-week-old male Wistar rats were purchased from Clea Japan (Tokyo, Japan) and housed individually in stainlesssteel cages at 22 °C with a 12-hour light-dark cycle. During the acclimatization period, all rats were fed a control diet for 3 days. Subsequently, the rats were randomly divided into three experimental groups comprising six rats each. The rats in two of the three groups were given free access to either a control (C group) or an iron-deficient (D group) diet. Rats in the third group (PF group) were pair-fed the control diet with the D group. The control diet was prepared according to the AIN-93G formula [10], and the iron-deficient diet was prepared in a similar way, except for the use of an iron-free AIN-93G mineral mixture. Throughout the experimental period, all rats had free access to deionized water. Urine was collected using metabolic cages with providing their respective diet over the last 24 h of the experimental period. After 4 weeks, all rats were sacrificed under anesthesia, and blood and femur samples were collected for analysis. Hemoglobin concentration was measured using a hemoglobin B-test kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). In addition, hearts were removed and weighed to assess heart enlargement, a sign of iron-deficiency anemia.

Measurement of bone turnover markers

Blood samples were centrifuged at 1,500 × g for 15 min after coagulation and the supernatants were collected to analyze the levels of serum osteocalcin, a bone formation marker. Serum and urine samples were stored at -80 °C until analysis. The serum osteocalcin concentration was measured using the rat osteocalcin/bone gla protein enzyme-linked immunosorbent assay system (GE Healthcare Japan Corporation, Tokyo, Japan). Urinary CTx was measured using the RatLapsTM enzyme-linked immunosorbent assay (Immunodiagnostic Systems Nordic A/S, Herlev, Denmark). Urinary DPD excretion was measured using the METRA DPD EIA kit (Quidel, San Diego, CA, USA). Urinary creatinine excretion was measured using the Jaffé reaction [11].

Measurement of femoral BMC and BMD

Left femur samples were stored in 70% ethanol at 4 °C until analysis. Subsequently, the BMC and BMD of the femur were measured using dual-energy X-ray absorptiometry (DSC-600EX; Hitachi Aloka Medical, Ltd., Tokyo, Japan).

Isolation of total RNA and subsequent real-time PCR to measure bone metabolism-related mRNA expression

Right femur samples were stored at –80 °C until analysis. The samples were homogenized in TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) using a Precellys homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) in ceramic

bead-containing tubes for two 20 s cycles at 7,500 rpm. Total RNA was isolated from the lysate using the Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Complimentary DNA was synthesized using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific). Specific primers were used for the analysis of insulin-like growth factor-1 (IGF-1) (Igf1: forward 5'- TTCAGTTCGTGTGTGGACCAAG-3', reverse 5'- GATCACAGCTCCGGAAGCAA-3'), bone morphogenetic protein 2 (BMP2) (Bmp2: forward 5'-ACCGTGCTCAGCTTCCATCAC-3', reverse 5'-TTCCTGCATTTGTTCCCGAAA-3'), Runx2 (Runx2: forward 5'-GCACCCAGCCCATAATAGA-3', reverse 5'-TTGGAGCAAGGAGAACCC-3'), Osterix (Sp7: forward 5'-GCCTACTTACCCGTCTGACTTT-3', reverse 5'-GCCCACTATTGCCAACTGC-3'), osteopontin (Spp1: forward 5'-TGAGACTGGCAGTGGTTTGC-3', reverse 5'-CCACTTTCACCGGGAGACA-3'), osteocalcin (Bglap: forward 5'-GAGCTAGCGGACCACATTGG-3', reverse 5'-CCTAAACGGTGGTGCCATAGA-3'), collagen type I alpha 1 chain (Col1a1: forward 5'-TTCACCTACAGCACGCTTGTG-3', reverse 5'-GATGACTGTCTTGCCCCAAGTT-3'), TRAP (Acp5: forward 5'-CAGCCTTATTACCGTTTGC-3', reverse 5'-GAATTGCCACAGCATCAC-3'), cathepsin K (Ctsk: forward 5'-TGTCTGAGAACTATGGCTGTGG-3', reverse 5'-ATACGGGTAACGTCTTCAGAG-3'), and β-actin (Actb: forward 5'-AGCCATGTACGTAGCCATCCA-3', reverse 5'-TCTCCGGAGTCCATCACAATG-3') [12-16]. Their amplification specificity was confirmed by melting curve analysis. mRNA expression levels were normalized to that of the housekeeping gene, Actb.

Statistical analysis

Results are expressed as the mean \pm standard error for each group of 6. All statistical analyses were performed using SPSS Statistics ver.23 (IBM Corp., Armonk, NY, USA). One-way analysis of variance, followed by the Fisher's protected least significant difference test, was performed to evaluate significant differences among the groups. Differences were considered statistically significant at p < 0.05.

Results

Body weight, food intake, and indicators of iron deficiency

Final body weight and food intake were significantly lower in the D and PF groups than in the C group (**Table 1**), and these parameters did not differ significantly between the D and PF groups. Hemoglobin concentration was significantly lower and heart weight was significantly higher in the D group than in the C and PF groups. Hemoglobin concentration and heart weight did not differ significantly between the C and PF groups.

 Table 1.
 Body weight, food intake, and indicators of iron deficiency in the C, D, and PF groups.

	С	D	PF
Initial body weight (g)	51.1 ± 1.4	51.1 ± 0.7	50.9 ± 1.2
Final body weight (g)	$240.5\pm8.0^{\text{a}}$	$167.0\pm6.1^{\rm b}$	$183.9\pm2.8^{\rm b}$
Food intake (g/4 weeks)	$422.3\pm12.6^{\text{a}}$	$314.4\pm10.8^{\rm b}$	$312.6\pm0.3^{\rm b}$
Hemoglobin concentration (g/dL)	$12.0\pm0.4^{\circ}$	$3.4\pm0.2^{\mathrm{b}}$	12.6 ± 0.2^{a}
Heart weight (g/100g body weight)	$0.29\pm0.01^{\rm b}$	$0.64\pm0.05^{\rm a}$	$0.29\pm0.01^{\rm b}$

C, control group; D, iron-deficient group; PF, pair-fed group. Data are presented as mean \pm standard error for each group (n = 6).

^{a,b}The different letters denote significant differences, p < 0.05.

Bone turnover markers and femoral BMC and BMD

Serum osteocalcin concentration was significantly lower in the D group than in the C and PF groups and was significantly lower in the PF group than in the C group (**Table 2**). Urinary CTx excretion was significantly higher and urinary DPD excretion was significantly lower in the D group than in the C and PF groups. There were no significant differences in urinary CTx and DPD excretion between the C and PF groups. Femoral BMC and BMD were significantly lower in the D group than in the C and PF groups, and significantly lower in the PF group than in the C group.

Table 2.Bone turnover markers and femoral bone mineral content and bone mineral density in the C, D,
and PF groups.

	С	D	PF
Serum osteocalcin (ng/mL)	130.7 ± 1.6^{a}	$89.5 \pm 2.9^{\circ}$	116.3 ± 2.3^{b}
Urine CTx (μg/mmol creatinine)	$45.6 \pm 2.0^{\rm b}$	$70.4 \pm 8.1^{\circ}$	$30.8\pm4.2^{\rm b}$
Urine DPD (nmol/mmol creatinine)	$621.2 \pm 58.6^{\circ}$	$396.4\pm27.2^{\mathrm{b}}$	$545.5\pm35.5^{\text{a}}$
Femoral BMC (mg)	$143.8\pm1.7^{\text{a}}$	$122.9 \pm 3.8^{\circ}$	$134.7\pm2.7^{\rm b}$
Femoral BMD (mg/cm ²)	90.7 ± 1.0^{a}	$78.5 \pm 1.0^{\circ}$	$87.0\pm1.0^{\rm b}$

C, control group; D, iron-deficient group; PF, pair-fed group; CTx, C-terminal telopeptide of type I collagen; DPD, deoxypyridinoline; BMC, bone mineral content; BMD, bone mineral density. Data are presented as mean \pm standard error for each group (n = 6).

^{a,b,c}The different letters denote significant differences, p < 0.05.

Femoral bone formation-related mRNA expression

Femoral mRNA expression levels of *Igf1*, *Bmp2*, *Runx2*, *Sp7*, *Spp1*, *Bglap*, and *Col1a1* were significantly lower in the D group than in the C and PF groups (**Table 3**). *Bglap* expression was significantly lower in the PF group than in the C group. There were no significant differences in the expression of *Igf1*, *Bmp2*, *Runx2*, *Sp7*, *Spp1*, or *Col1a1* between the C and PF groups.

	С	D	PF
Igf1	$1.00\pm0.05^{\text{a}}$	$0.65\pm0.01^{\rm b}$	$0.86\pm0.09^{\text{a}}$
Bmp2	1.00 ± 0.06^{a}	$0.59\pm0.05^{\rm b}$	$1.04\pm0.06^{\rm a}$
Runx2	1.00 ± 0.15^{a}	$0.56\pm0.04^{\rm b}$	$1.25\pm0.12^{\text{a}}$
Sp7	1.00 ± 0.15^{a}	$0.38\pm0.04^{\rm b}$	$1.38\pm0.15^{\rm a}$
Spp1	$1.00\pm0.05^{\circ}$	$0.67\pm0.03^{\rm b}$	$0.98\pm0.03^{\text{a}}$
Bglap	$1.00\pm0.03^{\text{a}}$	$0.60\pm0.04^{\rm c}$	$0.88\pm0.04^{\rm b}$
Col1a1	1.00 ± 0.07^{a}	$0.55\pm0.03^{\rm b}$	$0.93\pm0.04^{\rm a}$

 Table 3.
 Bone formation-related mRNA expression in the femur in the C, D, and PF groups.

C, control group; D, iron-deficient group; PF, pair-fed group. Data are presented as mean \pm standard error for each group (n = 6). The value of the C group was considered to be 1.00.

^{a,b,c}The different letters denote significant differences, p < 0.05.

Femoral bone resorption-related mRNA expression

Femoral *Acp5* mRNA expression was significantly lower in the D group than in the C and PF groups (**Table 4**). There was no significant difference in *Acp5* expression between the C and PF groups. Femoral *Ctsk* mRNA expression was significantly lower in the D and PF groups than in the C group. There was no significant difference in *Ctsk* expression between the D and PF groups.

Table 4. Bone resorption-related mRNA expression in the femur in the C, D, and PF g	groups
--	--------

	С	D	PF
Acp5	$1.00\pm0.09^{\text{a}}$	$0.50\pm0.03^{\rm b}$	$0.92\pm0.11^{\circ}$
Ctsk	$1.00\pm0.10^{\text{a}}$	$0.63\pm0.05^{\rm b}$	$0.78\pm0.03^{\rm b}$

C, control group; D, iron-deficient group; PF, pair-fed group. Data are presented as mean \pm standard error for each group (n = 6). The value of the C group was considered to be 1.00.

^{a,b}The different letters denote significant differences, p < 0.05.

Discussion

Undernutrition is a cause of bone loss, and dietary iron deficiency reduces BMD and bone strength. In this study, dietary iron deficiency decreased hemoglobin concentration and femoral BMC and BMD, as previously reported [2-4]. Since hemoglobin concentration and femoral BMC and BMD were lower in the D group than in the PF group, dietary iron deficiency could be the dominant cause of anemia and bone loss, independent of decreases in food intake and body weight, similar to our previous studies [3, 4].

Decreased osteoblastic bone formation is one of the reasons dietary iron deficiency induces lower BMC and BMD. The serum P1NP concentration is decreased in iron-deficient rats [7]. Furthermore, in this study, the serum osteocalcin concentration was significantly lower in the D group than in the C and PF groups, as previously reported [3, 4]. These results suggest that dietary iron deficiency causes a decline in bone formation. Dietary iron deficiency decreases the serum 1,25-dihydroxyvitamin D₃ concentration, which is caused by a decline in renal 25-hydroxyvitamin D-1 α -hydroxylase activity [4]. Thus, although we did not measure the serum 1,25-dihydroxyvitamin D₃ concentration in this study, it is possible that a lower serum 1,25-dihydroxyvitamin D₃ concentration due to dietary iron deficiency might lead to decreased bone formation.

Runx2 and Osterix are essential transcription factors for osteoblast differentiation [8, 9]. In this study and our previous study [4], dietary iron deficiency decreased femoral *Runx2* and *Sp7* mRNA expression, indicating that decreased *Runx2* and *Sp7* mRNA expression may cause a decrease in osteoblast differentiation in iron-deficient rats. Furthermore, Runx2 regulates the expression of several osteoblastic genes, including *Spp1*, *Bglap*, and *Col1a1* [17]. In this study, femoral *Spp1*, *Bglap*, and *Col1a1* mRNA expression levels were significantly lower in the D group than in the C and PF groups, suggesting that a decrease in Runx2 expression downregulates the expression of these osteoblastic genes. However, decreased osteoblast number could reduce the expression of osteoblastic genes without downregulating these gene expression per osteoblast. Therefore, further studies may be needed to clarify the downregulation of osteoblastic genes in osteoblastic cells of iron-deficient rats.

In this study, we measured femoral *Bmp2* and *Igf1* mRNA expression. BMP2 is a potent inducer of bone formation that stimulates osteoblast differentiation and regulates Runx2 and Osterix expression [18, 19]. IGF-1 upregulates osteogenic-related genes such as *Bmp2*, *Runx2*, *Spp1*, and *Bglap* [20]. Furthermore, BMP2 and IGF-1 regulate Osterix expression during osteoblast lineage progression [21]. In this study, femoral *Bmp2* and *Igf1* mRNA expression levels were significantly lower in the D group than in the C and PF groups. These results suggest that dietary iron deficiency decreases BMP2 and IGF-1 expression, leading to reduced Runx2 and Osterix expression. Furthermore, 1,25-dihydroxyvitamin D₃ enhances IGF-1 production in osteoblast-like cells *in vitro* [22]. Therefore, we hypothesized that decreased 1,25-dihydroxyvitamin D₃ due to dietary iron deficiency reduces

IGF-1 expression, which, in part, is related to a decrease in osteoblast differentiation. Consequently, our results revealed that dietary iron deficiency decreased IGF-1 and BMP2 expression, which decreased bone formation through downregulation of osteoblastogenesis-related genes.

Dietary iron deficiency decreased urinary DPD excretion in this study, as previously reported [2, 3]. Furthermore, according to previous studies based on bone histomorphometry, osteoclast numbers are reduced in iron-deficient rats [3, 4]. Therefore, we hypothesized that dietary iron deficiency would reduce bone resorption. In this study, femoral *Acp5* and *Ctsk* mRNA expression levels were significantly lower in the D group than in the C group, supporting the finding of decreased osteoclastic bone resorption due to dietary iron deficiency. *In vitro* studies have shown that iron chelation by deferoxamine inhibits osteoclast differentiation in murine bone marrow macrophages [23, 24]. Finally, our results showed that dietary iron deficiency inhibited osteoclastic bone resorption. However, Díaz-Castro *et al.* demonstrated that dietary iron deficiency increased serum CTx and TRAP concentrations in rats [7]. The reason for this discrepancy is unknown, and detailed examinations are necessary to clarify the conditions under which dietary iron deficiency induces increased bone resorption.

Conclusion

We examined the effects of dietary iron deficiency on bone metabolism-related mRNA expression in rats and found decreased femoral *Igf1, Bmp2, Runx2, Sp7, Spp1, Bglap, Col1a1, Acp5*, and *Ctsk* mRNA expression. Our study revealed that dietary iron deficiency reduced the expression of BMP2 and IGF-1 as well as decreased bone formation. The decreased expression of BMP2 and IGF-1 by iron deficiency could decrease osteoblast differentiation and osteoblastic bone formation. Furthermore, osteoclastic gene expression indicated a decrease in osteoclastic bone resorption in iron-deficient rats.

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