Metallomics Research 2022 (2) #MR202204

Regular article https://metallomicsresearch.brte.org

Changes in the Renal Copper Concentration in Rats as a Function of Phosphorus Intake

Munehiro Yoshida*, Suzuno Ikeda, Ryota Hosomi, Kenji Fukunaga

Laboratory of Food and Nutritional Sciences, Faculty of Chemistry, Materials and Bioengineering, Kansai University

Summary

We have found that the renal copper concentrations are markedly decreased in rats fed a low-phosphorus diet or administered phosphate binders. To clarify the relationship between phosphorus intake and renal copper concentration, we examined the renal copper concentrations in rats fed three types of diet with different phosphorus content. Eighteen 4-week-old male Wistar rats were divided into 3 groups and fed either a low phosphorus diet (phosphorus concentration, 0.15%), a control diet (phosphorus concentration, 0.3%), or a high phosphorus diet (phosphorus concentration, 0.6%) for 4 weeks. The serum phosphorus concentration reflected the phosphorus intake, with the highest values in the high phosphorus diet group, control group, and low phosphorus diet group, in that order. In the liver, the low phosphorus diet group had a significantly lower copper concentration than the other two groups, but the difference was not large. The kidney copper concentrations were remarkably different among the groups, with the highest values in the high phosphorus diet group, control group, and low phosphorus diet group, in that order. There were no differences in the iron, zinc, and manganese concentrations among the groups, except for a higher liver iron concentration in the low phosphorus diet group. These results indicate that the renal copper concentration increases or decreases specifically in response to changes in the phosphorus intake.

Key words: renal copper, phosphorus intake, interaction between minerals

Statements about COI: The authors have no conflicts of interest directly relevant to the content of this article.

*Correspondence:
3-3-35 Yamate, Suita, Osaka 564-8680, Japan
Tel: +81 6 6368 0970 (cell phone number: +81 90 9990 1853)
E-mail: hanmyou4@kansai-u.ac.jp

Received: May 24, 2022
Accepted: August 01, 2022
Released online: August 18, 2022

Introduction

Phosphorus is an essential element in the formation of bone salts and cell membranes. It is also a material found in several nucleotides and high-energy phosphate compounds and is a major intracellular electrolyte. Thus, its excess or deficiency induces various health disorders [1]. However, the risk of specific dietary phosphorus deficiency is low because much of the phosphorus in the diet is protein bound [2]. In contrast, the high use of phosphate compounds in food additives may lead to excessive phosphorus intake [3].

Inadequate urinary excretion of phosphorus, such as in chronic renal failure, may increase the serum phosphorus level and cause ectopic calcification due to the binding of phosphate

with calcium [4]. If this calcification occurs in the coronary or cerebral arteries, it can induce fatal ischemic disease. For this reason, patients with chronic renal failure should take phosphate binders to inhibit the absorption of phosphorus in the diet and prevent hyperphosphatemia [5].

Because phosphorus is bound to dietary proteins, it is difficult to prepare a severely low phosphorus diet. But a phosphate binder can be administered to experimental animals to induce a low phosphorus state [6]. We found that the administration of phosphate binders, such as lanthanum carbonate or iron citrate, to normal rats decreased the copper concentration in the kidney [7,8]. This decrease in the renal copper concentration was also observed in rats fed a mildly low phosphorus diet [9], suggesting that the low phosphorus state caused the decrease, not the effect of the metals used in the phosphate binders.

No studies have been found to explain the phenomenon of a low phosphorus state reducing the kidney copper levels. Nor has the interaction between phosphorus and copper been examined in the first place. In the present study, to clarify the relationship between phosphorus intake and renal copper concentration, the effect of high phosphorus feeding on the renal copper concentration was examined.

Materials and methods

Animal feeding

The experimental protocol followed the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and was reviewed and approved by the Animal Ethics Committee of Kansai University (Approval No. 2005).

Eighteen 4-week-old male Wistar rats (SHIMIZU Laboratory Supplies Co., Kyoto) were divided into 3 groups. One group (control group) was fed a basal AIN93G diet (control diet) with a phosphorus concentration of 0.3% [10], and the other two groups (low phosphorus group and high phosphorus group) were fed a low phosphorus diet with a phosphorus concentration of 0.15% and a high phosphorus diet with a phosphorus concentration of 0.6%, respectively. The composition of each diet is summarized in **Table 1**. The low phosphorus diet was prepared by replacing potassium dihydrogen phosphate in the AIN93G

Table 1. | Composition of the experimental diet

	Low P diet	Control diet	High P diet
Ingredients (g/kg)			
Casein	200	200	200
Sucrose	100	100	100
Soybean oil	70	70	70
Corn starch	391.84	391.84	385.88
Gelatinized corn starch	132	132	132
Phosphorus-free mineral mixture[1)]	35	–	–
AIN93G mineral mixture	–	35	35
AIN93G vitamin mixture	10	10	10
Cellulose	50	50	50
Choline bitartrate	2.5	2.5	2.5
L-Cystine	3	3	3
NaH_2PO_4	–	–	11.62
NaCl[2)]	5.66	5.66	–
Phosphorus content (%)[3)]	0.15	0.30	0.60
Chloride ion (Cl^-) content (%)	0.68	0.50	0.16

[1)] Prepared by removing KH_2PO_4 from the AIN93G mineral mix and adding KCl to equalize the potassium concentration.
[2)] Added to equalize the sodium concentration.
[3)] Contains phosphorus derived from casein.

mineral mixture with potassium chloride, and the high phosphorus diet was prepared by adding sodium dihydrogen phosphate to the AIN93G diet. Sodium chloride was also added to the low phosphorus and control diets to equalize the sodium intake of each group. As a result, the chloride ion concentrations in the diets were highest in the low phosphorus diet, the control diet, and the high phosphorus diet, in that order.

During the feeding period, the animals ingested the diets and water (tap water) *ad libitum*. After feeding for 4 weeks, the livers, kidneys, and blood were collected under isoflurane (Fujifilm Wako Pure Chemical Co., Tokyo) anesthesia. Some of the blood was centrifuged at 1,500 x g for 15 minutes to obtain serum. The livers and kidneys were frozen in liquid nitrogen and stored at −30°C until analysis.

Analysis

One kidney and approximately 1 g of liver were heated with 5 mL nitric acid until there were no solids. The obtained solution was diluted with pure water and filtrated through a 0.45 μm filter, and zinc, iron, copper, and manganese were determined using an atomic absorption spectrophotometer (AA-7000, Shimadzu, Kyoto) or inductively coupled plasma mass spectrometer (ICPMS-2030, Shimadzu, Kyoto). In the analysis with ICPMS, ^{45}scandium was used as an internal standard. The other kidney was homogenized in 10 times volume of saline and centrifuged (105,000 x g, 60 minutes) to separate the soluble fraction and precipitate, and the respective copper concentrations were determined. Serum zinc and copper were measured using a commercial kit (Metalloassay LS, Metallogenics Co., Chiba).

Whole blood hemoglobin and serum total protein, triacylglycerol, total cholesterol, urea nitrogen, uric acid, chloride ion, calcium, magnesium, inorganic phosphorus, iron and total iron binding capacity were determined by Japan Medical Laboratory Co. (Kaizuka).

For each measurement, the differences among the groups were tested by one-way ANOVA followed by the Tukey's multiple comparison test. GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego) was used as the statistical analysis application.

Results

The body weights and organ weights at the end of the feeding period, as well as water consumption and feed efficiency during the feeding period are summarized in **Table 2**. In the low phosphorus group, body weight and feed efficiency were lower than in the other two groups. However, this group had a higher kidney weight per body weight and drank more water during the feeding period. Although the exact amounts were not measured, the low phosphorus group also had a higher urine output. On the other hand, the high phosphorus group did not differ from the control group in terms of the body and organ weights.

The serum biochemical parameters and hemoglobin concentrations are shown in **Table 3**. The serum inorganic phosphorus concentrations reflected the dietary phosphorus concentrations and were highest in the high phosphorus group, control group, and low phosphorus group, in that order. No differences were observed between the high phosphorus group and the control group for the other parameters. On the other hand, the low phosphorus group had lower triacylglycerols and higher uric acid, calcium, and hemoglobin values than the other two groups. Serum copper also tended to be lower in the low phosphorus group, but the difference was not significant.

The iron, zinc, copper, and manganese concentrations in the liver and kidneys are shown in **Table 4**. In the liver, a significant accumulation of iron was observed in the low phosphorus group. The copper concentrations tended to be higher with a higher phosphorus intake, with significant differences between the high and low phosphorus groups. In the kidneys, on the other hand, there were notable differences in the copper concentrations among the groups. That is, the low phosphorus group had the lowest kidney copper concentration, while the high phosphorus group had significantly higher values than the control group. In other words, the kidney copper concentrations were lower when the phosphorus intake was low and higher when the phosphorus intake was high.

The kidneys were divided into soluble and insoluble fractions, and their respective copper concentrations were measured. As shown in **Figure 1**, in both fractions, the copper concentrations were significantly higher with increasing phosphorus intake.

Table 2. | Body weight, liver and kidney weights, and feed efficiency

	Low P	Control	High P	ANOVA
Body weight (g)	245.0 ± 8.1[a]	291 1 ± 3.1[b]	295.8 ± 4.0[b]	p<0.001
Feed efficiency[1)]	0.411 ± 0.018[a]	0.507 ± 0.009[b]	0.473 ± 0.008[b]	p<0.001
Water intake (mL/d)	41.7 ± 2.6[b]	36.3 ± 2.5[ab]	31.6 ± 2.7[a]	p=0.047
Liver weight (g/100 g body weight)	4.09 ± 0.14	4.05 ± 0.06	4.21 ± 0.10	NS[2)]
Kidney weight (g/100 g body weight)	0.82 ± 0.02[b]	0.74 ± 0.01[a]	0.71 ± 0.01[a]	p<0.001

Values are means ± SEM (n=6). Tukey's multiple comparison was performed when ANOVA was significant (p<0.05); means in the same row not sharing a common superscript differ significantly (p<0.05) in the multiple comparison.
[1)] Calculated from the formula: (body weight gain during the feeding period (g)) / (total food intake (g)).
[2)] NS: not significant

Table 3. | Serum biochemical parameters and hemoglobin concentration

	Low P	Control	High P	ANOVA
Serum biochemical parameters				
Total protein (mg/dL)	5.3 ± 0.1	5.3 ± 0.1	5.4 ± 0.1	NS[1)]
Triacylglycerol (mg/dL)	52 ± 5[a]	88 ± 16[b]	73 ± 15[b]	p=0.002
Total cholesterol (mg/dL)	76 ± 4	75 ± 5	79 ± 4	NS
Urea nitrogen (mg/dL)	21 ± 2	19 ± 1	18 ± 1	NS
Uric acid (mg/dL)	1.23 ± 0.06[b]	0.78 ± 0.09[a]	0.77 ± 0.05[a]	p<0.001
Chloride ion (mEq/L)	100 ± 1	101 ± 1	100 ± 1	NS
Calcium (mg/dL)	12.55 ± 0.21[b]	10.65 ± 0.14[a]	10.85 ± 0.06[a]	p<0.001
Magnesium (mg/dL)	1.82 ± 0.05	1.87 ± 0.03	1.85 ± 0.06	NS
Inorganic phosphorus (mg/dL)	4.53 ± 0.37[a]	7.02 ± 0.30[b]	8.13 ± 0.20[c]	p<0.001
Iron (μg/dL)	289 ± 18	288 ± 16	269 ± 23	NS
Transferrin saturation (%)	62.4 ± 5.2	58.2 ± 3.4	50.7 ± 4.3	NS
Zinc (μg/dL)	237 ± 11	231 ± 13	226 ± 19	NS
Copper (μg/dL)	119 ± 9	155 ± 18	152 ± 27	NS
Hemoglobin (g/dL)	14.0 ± 0.2[b]	13.5 ± 0.1[a]	13.5 ± 0.1[a]	p=0.007
Hematocrit value (%)	40.8 ± 0.6	39.9 ± 0.3	39.5 ± 0.3	NS

Values are means ± SEM (n=6). Tukey's multiple comparison was performed when ANOVA was significant (p<0.05); means in the same row not sharing a common superscript differ significantly (p<0.05) in the multiple comparison.
[1)] NS: not significant

Table 4. | Iron, zinc, copper, and manganese concentrations (μg/g) in liver and kidney

	Low P	Control	High P	ANOVA
Liver				
Iron	98.8 ± 5.5[b]	81.5 ± 3.1[a]	83.4 ± 3.6[ab]	p=0.020
Zinc	27.0 ± 0.5	26.9 ± 0.6	27.2 ± 0.9	NS[1)]
Copper	3.29 ± 0.05[a]	3.44 ± 0.05[ab]	3.53 ± 0.07[b]	p=0.030
Manganese	2.79 ± 0.08	2.94 ± 0.31	2.44 ± 0.15	NS
Kidney				
Iron	45.9 ± 1.6	46.9 ± 1.2	47.9 ± 0.6	NS
Zinc	19.7 ± 1.3	21.4 ± 1.1	21.3 ± 0.7	NS
Copper	4.26 ± 0.13[a]	12.19 ± 0.45[b]	17.75 ± 1.21[c]	p<0.001
Manganese	1.07 ± 0.05	1.18 ± 0.09	0.99 ± 0.03	NS

Values are means ± SEM (n=6). Tukey's multiple comparison was performed when ANOVA was significant (p<0.05); means in the same row not sharing a common superscript differ significantly (p<0.05) in the multiple comparison.

[1)] NS: not significant

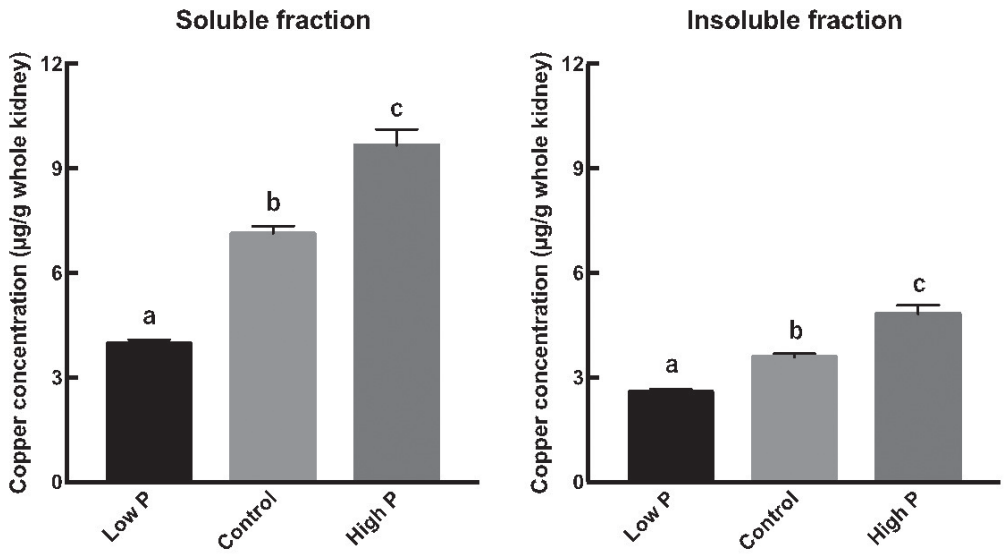

Figure 1. | Copper concentrations in soluble and insoluble fractions of the kidney
The height of the box and the length of the vertical line show the mean and SEM (n=6), respectively. Means in the same frame not sharing a common superscript differ significantly (p<0.05) in the Tukey's multiple comparison.

Discussion

In the present experiment, potassium dihydrogen phosphate was replaced with potassium chloride in the mineral mixture for the preparation of the low phosphorus diet. Because sodium dihydrogen phosphate was used to prepare the high phosphorus diet, sodium chloride was added to the control and low phosphorus diets to equalize the sodium intake. As a result, the concentration of chloride ions in the diets was in the following order: low phosphorus diet > control diet > high phosphorus diet (**Table 1**).

Recently, European Food Safety Authority (EFSA) has calculated the Dietary Reference Values (DRV) for chloride from the DRV for sodium, based on the assumption that chloride ions are consumed as sodium chloride [11]. This EFSA document does not specify the effects of excessive intake of chloride ions alone, independent of sodium or potassium. Since very little chloride are excreted in the feces [12] and are mostly absorbed in the gastrointestinal tract [11], it is possible that the blood concentration of chloride ions is maintained at a constant level by increasing urinary excretion rather than reducing absorption.

The water consumption and kidney weights of the rats fed the low phosphorus diet were significantly higher than those of the other two groups (**Table 2**). Although the exact amounts were not measured, it was observed that this group also produced more urine than the other two groups. On the other hand, there was no difference in the serum chloride ion concentrations among the three groups (**Table 3**) despite the large difference in chloride ion intake. These results suggest that rats in the low phosphorus group drank large amounts of water to excrete excess chloride ions in the urine, resulting in the accumulation of more water in the kidneys and an increase in their weight.

The main focus of the current experiment was to determine whether the changes in the kidney copper concentration that occurred in rats on the low-phosphorus diet were also observed on the high-phosphorus diet; in other words, whether increases or decreases in the phosphorus intake led directly to increases or decreases in the kidney copper concentrations. The results, as shown in **Table 4**, indicate that the renal copper concentrations do indeed increase or decrease in dependence on phosphorus intake. That is, the renal copper concentrations decreased when the phosphorus intake was low and increased when the phosphorus intake was high. To date, there have been no reported cases of such a phenomenon.

The body weights were lower in the low phosphorus diet group than in the other two groups. Feed efficiency was also decreased, suggesting that energy metabolism was not sufficiently maintained due to phosphorus deficiency at a dietary concentration of 0.15%. It has been reported that rats fed a low-phosphorus diet (0.2% phosphorus) show decreased serum phosphorus concentration, decreased bone mineralization rate, and growth inhibition [13]. The growth inhibition observed in rats fed a low-phosphorus diet (0.15%) in this study may be due to delayed bone formation resulting from decreased bone mineralization rate, in addition to decreased energy metabolism caused by phosphorus deficiency.

We have observed a decrease in the renal copper concentration in 8-week-old rats on a low-phosphorus diet or phosphate-binder without changes in the body weight or kidney weight [7-9]. Therefore, it is unlikely that the decrease in the renal copper concentration observed in rats on a low phosphorus diet is a secondary effect of growth inhibition.

On the other hand, in an experiment in which rats were given excess phosphorus, renal hypertrophy and calcification were observed when the dietary concentrations exceed about 1% [14]. Since no such abnormalities were observed at the 0.6% dietary phosphorus concentration in the present study, it can be concluded that no damage occurred as a result of excessive phosphorus intake. Thus, the increase or decrease in the renal copper concentration with changes in the phosphorus intake appears to be a primary effect of the increase or decrease in phosphorus intake.

Thus, in the present experiment, the increase in renal copper concentration associated with a high phosphorus diet was not particularly damaging to renal function. However, since copper accumulation in tissues can cause tissue damage via the Fenton reaction, the health effects of copper accumulation at higher doses of phosphorus than in the present study should be examined. In addition, the effects of decreased renal copper concentrations due to a low-phosphorus diet on the physiological functions of copper, such as the activity of copper-containing enzymes, should also be examined.

The liver copper levels were significantly lower only when fed a low phosphorus diet (**Table 4**). The serum copper concentrations also tended to decrease in the low phosphorus group, although not significantly (**Table 3**). Although these changes are minor compared to the renal concentrations, they may indicate a change in whole-body copper dynamics as phosphorus intake increases or decreases.

Phosphorus homeostasis involves intestinal absorption, bone absorption and formation, renal excretion, and renal tubular reabsorption. These are regulated by the parathyroid hormone and 1,25-dihydroxy vitamin D [15]. A marked decrease in the

urinary excretion of phosphorus was observed in rats with hypophosphatemia induced by the administration of phosphate-binders [7,8], suggesting that renal reabsorption of phosphorus may play an important role in phosphorus homeostasis. Phosphorus reabsorption in the renal tubules is regulated by fibroblast growth factor 23 (FGF23), and an increase in the serum phosphorus concentration increases the serum FGF23 concentration, while a decrease in the phosphorus concentration decreases the FGF23 concentration [16]. Such a change in the FGF23 concentration in response to an increase or decrease in the serum phosphorus concentration may have influenced the increase or decrease in the renal copper concentration. However, the relationship between FGF23 and urinary copper excretion is unknown. In addition, urine is not a major route of copper excretion, as copper is excreted *via* bile. Therefore, it is unlikely that FGF23 is involved in changes in the renal copper concentration.

Phosphate ions form insoluble salts with many metal ions. The significantly higher serum calcium concentration and the significantly higher liver iron and hemoglobin concentrations in the low phosphorus group (**Tables 3 and 4**) may indicate that the low phosphorus diet decreased the phosphate concentration in the gastrointestinal tract and increased the amounts of soluble calcium and iron that were absorbed.

Copper ions will also form insoluble salts with phosphate ions. If such insoluble salts are formed in the blood, they may not be filtered by the glomeruli of the kidney and may accumulate in the kidney. However, when the kidneys were divided into soluble and insoluble fractions and the copper concentrations were measured, they both changed in response to phosphorus intake (**Figure 1**). Furthermore, such insoluble salts could also be formed between iron or zinc ions and phosphate ions, but only copper showed significant changes in the kidney concentration. In other words, it is difficult to establish a mechanism by which insoluble salts of phosphate and copper were formed in the blood and accumulated in the kidneys.

As described above, changes in phosphorus intake may affect calcium and iron absorption. In particular, there have been several reports on the interaction between iron and copper and excess iron above the required level has been reported to inhibit copper absorption [17,18]. Accordingly, the present results may be mediated by changes in iron absorption rather than a direct effect of phosphorus. Therefore, it is necessary to reexamine copper concentrations in various organs when iron doses are varied, including changes in phosphorus in the body.

Acknowledgment

This study was supported by Grant-in-Aid for Scientific Research (22K055180).

References

[1] Takeda E, Yamamoto H, Yamanaka-Okumura H, Taketani Y: Dietary phosphorus in bone health and quality of life. Nutr Rev 70: 311-321, 2012.

[2] Chang AR, Anderson C: Dietary Phosphorus intake and the kidney. Annu Rev Nutr 37: 321-346, 2017.

[3] St-Jules DE, Goldfarb DS, Pompeii ML, Sevick MA: Phosphate additive avoidance in chronic kidney disease.Diabetes Spectr 30: 101-106, 2017.

[4] Tomson C: Vascular calcification in chronic renal failure. Nephron Clin Pract 93: c124-130, 2003.

[5] Ritz E: The clinical management of hyperphosphatemia. J Nephrol 18: 221-228, 2005.

[6] Yoshida M, Hosomi R, Kunimatsu M, Nakamura M, Fukunaga K, Kanda S, Nishiyama T: Phosphorus balance in rats with hypophosphatemia induced by lanthanum carbonate. Food Nutr Sci 3: 105-110, 2012.

[7] Yoshida M, Hosomi R, Noda H, Uemura K, Fukunaga K: Effect of lanthanum-induced low phosphorus status on tissue trace mineral contents. Proceedings of 15th International Conference on Heavy Metals in the Environment, Bargańska Ż, Beyer A, Klimaszewska K, Namieśnik J, Tobiszewski M, Rutkiewicz I (ed), Gdansk University of Technology, 2010, 870-872.

[8] Yoshida M, Yamakawa H, Yukawa N, Noguchi S, Fukunaga H, Nishiyama T: Transferrin saturation and tissue iron concentration in rats with high dose of ferric citrate. Biomed Res Trace Elem 24: 23-30, 2013.

[9] Noguchi S, Yukawa N, Fukunaga K, Nishiyama T, Yoshida M; Low phosphorus status contributes to high serum calcium and low renal copper levels in rats administered lanthanum. Trace Nutr Res 30: 31-34, 2013.

[10] Reeves PG, Nielsen FH, Fahey GC Jr: AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123: 1939-1951, 1993.

[11] European Food Safety Authority: Dietary reference values for chloride. EFSA J 17(9): 5779, 2019.

[12] Rose C, Parker A, Jefferson B and Cartmell E: The characterization of feces and urine: A review of the literature to inform advanced

treatment technology. Crit Rev Environ Sci Technol 45: 1827-1879, 2015.

[13] Baylink D, Wergedal J, Stauffer M: Formation, mineralization and resorption of bone in hypophosphatemic rats. J Clin Invest 50: 2519-2530, 1971.

[14] Hosomi R, Nakazawa C, Hagihara N, Fukunaga K, Yoshida M: Influence of the chemical form of phosphate salt and phosphorus level in the diet on kidney calcification and mineral balance in rats. J Jpn Health Med Assoc 29: 27-38, 2020.

[15] Bergwitz C, Jüppner H: Regulation of phosphate homeostasis by PTH, vitamin D, and FGF23. Annu Rev Med 61: 91-104, 2010.

[16] Gattineni J, Alphonse P, Zhang Q, Mathews N, Bates CM, Baum M: Regulation of renal phosphate transport by FGF23 is mediated by FGFR1 and FGFR4. Am J Physiol Renal Physiol 306: F351-358, 2014.

[17] Cohen NL, Keen CL, Lönnerdal B, Hurley L: Effects of varying dietary iron on the expression of copper deficiency in the growing rat: anemia, ferroxidase I and II, tissue trace elements, ascorbic acid, and xanthine dehydrogenase. J Nutr 115: 633-649, 1985.

[18] Ha JH, Doguer C, Wang X, Flores SR, Collins JF: High-iron consumption impairs growth and causes copper-deficiency anemia in weanling Sprague-Dawley rats. PLoS One 11(8): e0161033, 2016.