ORIGINAL

# Dietary polyphosphate has a greater effect on renal damage and FGF23 secretion than dietary monophosphate

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**Abstract : Phosphate (Pi)-containing food additives are used in several forms. Polyphosphate (PPi) salt has more harmful effects than monophosphate (MPi) salt on bone physiology and renal function. This study aimed to analyze the levels of parathyroid hormone PTH and fibroblast growth factor 23 (FGF23) and the expression of renal/intestinal Pi transport-related molecules in mice fed with an MPi or PPi diet. There were no significant differences in plasma Pi concentration and fecal Pi excretion levels between mice fed with the high-MPi and PPi diet. However, more severe tubular dilatation, interstitial fibrosis, and calcification were observed in the kidneys of mice fed with the high PPi diet versus the MPi diet. Furthermore, there was a significant increase in serum FGF23 levels and a decrease in renal phosphate transporter protein expression in mice fed with the PPi diet versus the MPi diet. Furthermore, the high MPi diet was associated with significantly suppressed expression and activity of intestinal alkaline phosphatase protein. In summary, PPi has a more severe effect on renal damage than MPi, as well as induces more FGF23 secretion. Excess FGF23 may be more involved in inflammation, fibrosis, and calcification in the kidney. J. Med. Invest. 69 : 173-179, August, 2022**

***Keywords*** **:** *Polyphosphate, FGF23, Kidney, Intestine*

## INTRODUCTION

Phosphorus is an essential nutrient for living organisms, not only as a component of teeth, bones, cell membranes, and nucleic acids, but also because of its involvement in the composition of ATP, regulation of protein functions, and acid-base balance. Normally, intestinal absorption, bone formation/resorption, and renal reabsorption respond to several factors to maintain phosphorus balance in the body (1-3). Phosphate (Pi) is filtered through the glomerulus and reabsorbed via the renal sodium phosphate (NaPi) cotransporters Npt2a and Npt2c, which are expressed on the apical side of the proximal tubular epithelial cells. Dietary phosphorus content, active vitamin D (1,25[OH]$_2$ D), phosphaturic hormones, parathyroid hormone (PTH), and fibroblast growth factor 23 (FGF23) are all significant regulators of Pi absorption and reabsorption (1-4). A low phosphorus diet increases intestinal Pi absorption and renal reabsorption. Moreover, active vitamin D is a regulator of intestinal Pi absorption. In contrast, intake of a high phosphorus diet is associated with a rapid response of PTH secretion from the parathyroid glands, followed by enhanced FGF23 secretion from the bone. PTH binds to PTH receptors expressed in the kidney to promote urinary Pi excretion, whereas FGF23 binds to α-Klotho and FGF receptor 1 in the kidney to inhibit Pi reabsorption, thereby promoting urinary Pi excretion. In addition, FGF23 inhibits intestinal Pi absorption by decreasing the synthesis of active vitamin D by suppressing the expression of 1alpha-hydroxylase and increasing the expression of 24hydroxylase (1-3). This axis of the intestine, kidney, bone, and parathyroid helps regulate plasma Pi levels. However, it is unclear how FGF23 secretion from bone is increased by a high phosphorus load and the setpoint of plasma Pi concentration.

In patients with chronic kidney disease, decreased renal function leads to decreased Pi excretion, resulting in hyperphosphatemia, which in turn leads to high levels of plasma PTH and FGF23. Hyperphosphatemia is a risk factor for ectopic calcification, secondary hyperparathyroidism, and cardiovascular disease (5, 6). However, even among healthy populations, phosphorus intake is associated with osteoporotic fractures, left ventricular mass, and mortality (7, 8). Thus, the management of phosphorus management is important not only in renal patients but also in healthy individuals with normal renal function (2, 5-7, 9).

Increased phosphorus intake is a worldwide problem (4, 10), notably because the typical diet contains a large amount of phosphorus. Dietary phosphorus is classified into organic and inorganic phosphorus. Organic phosphorus is found in animal products such as meat and fish, as well as in plant products such as soybeans and cereals (11, 12). Inorganic phosphorus (Pi salt) is commonly used in canned and boxed food processing to improve taste, texture, color, and cooking time, while also functioning as a preservative (4, 13). It is also added to meat and poultry products to retain moisture and protect flavor (4, 11, 13). The use of these additives by food manufacturers is increasing (13). There have been many reports on the effects of high phosphorus diets animal and human experiments (7, 8, 14-17). A high phosphorus diet was reported to increase serum and urinary Pi, decrease serum and urinary calcium, and induce renal structural damage, skeletal muscle atrophy, and vascular calcification (7, 8, 15, 18). Thus, the intake of Pi salt as a food additive, especially through the consumption of processed and ready-to-eat food, is of particular concern. Pi additives include monophosphate (MPi) and polyphosphate (PPi), which is a linear chain of Pi.

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PPi intake has been reported to affect renal function and bone structure to a greater degree compared to MPi intake (19, 20). However, the details of this effect have not yet been elucidated. This study aimed to analyze PTH and FGF23 levels and the expression of renal/intestinal Pi transport-related molecules in mice fed with MPi and PPi diets.

## EXPERIMENTAL PROCEDURES

*Experimental animals*

All experimental procedures involving animals were conducted in accordance with guidelines set by the Tokushima University School of Medicine. This study was also carried out in compliance with the ARRIVE guidelines. All procedures involving the use of animals were subject to approval from the ethics committee of Tokushima University School of Medicine (T2019-126).

Male and female C57B6/J mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Mice were provided free access to water and standard mouse chow containing 0.8% phosphorus (Oriental MF ; ORIENTAL YEAST CO., LTD, Osaka, Japan). For the dietary adaptation study, 5-week-old male mice were fed a test diet based on the modified AIN93G diet, referred to as the low Pi [LP] diet (0.02% Pi, 0.6% Pi, 1.2% Pi, or 1.8% Pi) for 1 or 5 weeks (21, 22). $KH_2PO_4$ and $K_5P_3O_{10}$ were used as phosphate additives for all forms of the MPi and PPi diets (i.e., 0.6%, 1.2%, or 1.8% Pi), respectively. Egg white was used as a protein source, and the LP diet does not contain any Pi salt.

*Metabolic cages to collect urine and fecal samples*

Mice were individually placed in metabolic cages at 10:00 AM for quantitative urine and fecal collection for 24 h with free access to food and water. Fecal samples were washed according to a modified protocol, as described previously (22, 23).

*Biochemical measurements*

Concentrations of Pi, Ca, and creatinine were determined using commercial kits (WAKO, Osaka, Japan). Concentrations of serum FGF23 and plasma PTH were determined using the FGF23 ELISA kit (KAINOS Laboratories, Tokyo, Japan) and intact PTH ELISA kit (Immunotopics Inc., San Clemente, CA, USA).

*RNA extraction, cDNA synthesis, and quantitative polymerase chain reaction (PCR)*

The total cellular RNA from the sampled mouse tissues was extracted and purified using ISOGEN (WAKO, Osaka Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized as described previously. Quantitative PCR was performed using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA). The reaction mixture consisted of 10 ml of SYBR Premix Ex Taq, ROX Reference Dye II (Perfect Real Time, TaKaRa Bio) with specific primers (Table 1).

*Protein sample purification and immunoblotting*

Brush border membrane vesicle (BBMV)s prepared using the $Ca^{2+}$ precipitation method were obtained from mouse intestine and kidneys and used for immunoblotting as described previously (21-23). Protein samples were heated at 95°C for 5 min in a sample buffer with the presence of 2-mercaptoethanol and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred by electrophoresis to Immobilon-P polyvinylidene difluoride (Millipore, Billerica, MA, USA) and treated with diluted antibodies. Signals were detected using Immobilon Western (Millipore).

*Antibodies*

Rabbit anti-Npt2a and Npt2c polyclonal antibodies were generated as described previously and used for immunoblotting (24, 25). Mouse anti-Npt2b polyclonal antibody was purchased from Alpha Diagnostic International, San Antonio, TX (26). Polyclonal rabbit anti-Akp3, Akp5, and Akp6 antiserum were used as described previously (22). Anti-actin monoclonal antibody (Millipore) was used as an internal control. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was utilized as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). The diluted antibodies for immunoblotting were as follows : anti-Npt2a (1 : 15000), anti-Npt2b (1 : 4000), anti-Npt2c (1 : 1500), anti-Akp3, Akp5, and Akp6 (1 : 50000), and anti-actin (1 : 10000).

*Measurement of intestinal alkaline phosphatase activity*

BBMVs were used for the measurement of alkaline phosphatase. For this study, the proximal intestine refers to the duodenum and the proximal part of the jejunum (22). Intestinal BBMVs (20 μg) were mixed with phosphatase assay reagent including 4 mM disodium-p-nitrophenyl Pi, 0.1 M glycine (pH 9.0), and 1 mM $MgCl_2$, then incubated at 37°C for 20 min. The reaction was stopped by adding 0.05 mM NaOH to the reaction mixture. A calibration curve was prepared with p-nitrophenol, and absorbance was measured at 400 nm. Alkaline phosphatase activity per microgram of protein was calculated.

*Histological analysis*

Mouse tissues were fixed with 4% paraformaldehyde overnight at 4°C and embedded in paraffin. Serial sections (5 μm thick) of several tissues were mounted on MAS-coated slides (Matsunami Glass IND, Ltd.). The sections were treated for Masson trichrome staining, hematoxylin, and von Kossa staining. Masson trichrome staining allowed for the observation of renal tubules and estimated fibrillization. The von Kossa staining for mineral deposits was performed by applying 5% silver nitrate to the

Table 1. Primers for real-time PCR

| Primer | Sense | Antisense |
|---|---|---|
| Npt2a | AGTCTCATTCGGATTTGGTGTCA | GCCGATGGCCTCTACCCT |
| Npt2c | TAATCTTCGCAGTTCAGGTTGCT | CAGTGGAATTGGCAGTCTCAA |
| IL-6 | ATCCAGTTGCCTTCTTGGGACTGA | TAAGCCTCCGACTTGTGAAGTGGT |
| Col1a1 | ACTACCGGGCCGATGATGCTAACG | CGATCCAGTACTCTCCGCTCTTCC |
| Lipocalin 2 | GGACCAGGGCTGTCGCTACT | GGTGGCCACTTGCACATTGT |
| GAPDH | CTGCACCACCAACTGCTTAGC | CATCCACAGTCTTCTGGGTG |

sections and exposing them to bright light for 60 min (27). The sections were slightly counterstained with hematoxylin.

*Statistical analysis*

Data are expressed as means ± SE. Differences among multiple groups were analyzed by analysis of variance followed by the Scheffe test. Differences between two experimental groups were established by analysis of variance followed by Student's *t* test. *P* values less than 0.05 were considered statistically significant.

## RESULTS

*Effects of monophosphate and polyphosphate diets on the biochemical data*

To measure food intake and urine and fecal biochemical data, mice were individually placed in metabolic cages after five weeks under the test diet. Mice fed with the LP diet (0.02% Pi) had significantly lower body weight than the other groups. However, there was no difference in body weight between the MPi and PPi diet groups (Figure 1A). No significant differences were observed between any test diets for food intake (Figure 1B).

Mice under the LP diet had significantly higher plasma Ca levels and lower fecal and urinary Ca excretion than the other groups (Figure 1C–1E). Furthermore, the LP diet group also had significant hypophosphatemia as well as low levels of fecal and urinary Pi excretion compared to the other groups (Figure 1F–1H). On the other hand, the difference in plasma and fecal, and urinary Ca and Pi parameters between the MPi and PPi diet groups was almost equivalent (Figure 1C–1H). However, when comparing the 1.2% MP and PP groups, urinary Pi excretion was significantly higher in the PP group than MP group (Figure 1H).

*Effects of monophosphate and polyphosphate diets on the kidneys*

In the MPi diet groups, no significant differences were observed for urinary volume and plasma creatinine level (Figure 2A, 2B). In the PPi diet, urine volume and plasma creatinine concentration increased significantly or tended to increase in the 1.8% group compared to the 0.6% and 1.2% groups (Figure 2A, 2B). Real-time PCR data for the inflammatory marker, interleukin (IL)-6, fibrosis marker, collagen, type I, alpha 1 (Col1a1), and tubular inflammatory marker (neutrophil gelatinase-associated Lipocalin 2 [NGAL]), are shown in Figures 2C–E. Both 1.8% MPi and PPi diets significantly or tended to increase the IL-6, collagen 1a1, and Lipocalin 2 mRNA compared to 0.6% and 1.2% groups. However, there was no difference in the mRNA levels of IL-6, collagen 1a1, and Lipocalin 2 between the MPi and PPi diets.

Afterwards, we observed renal fibrosis and calcification of the kidney of mice fed the test diet (Figure 2F, 2G). No significant differences were observed between the 0.6% MPi and PPi groups (Figure 2F-i, -iii, and 2G-i, -iv). In both 1.8% MPi and PPi diets, the proximal convoluted tubules demonstrated tubular

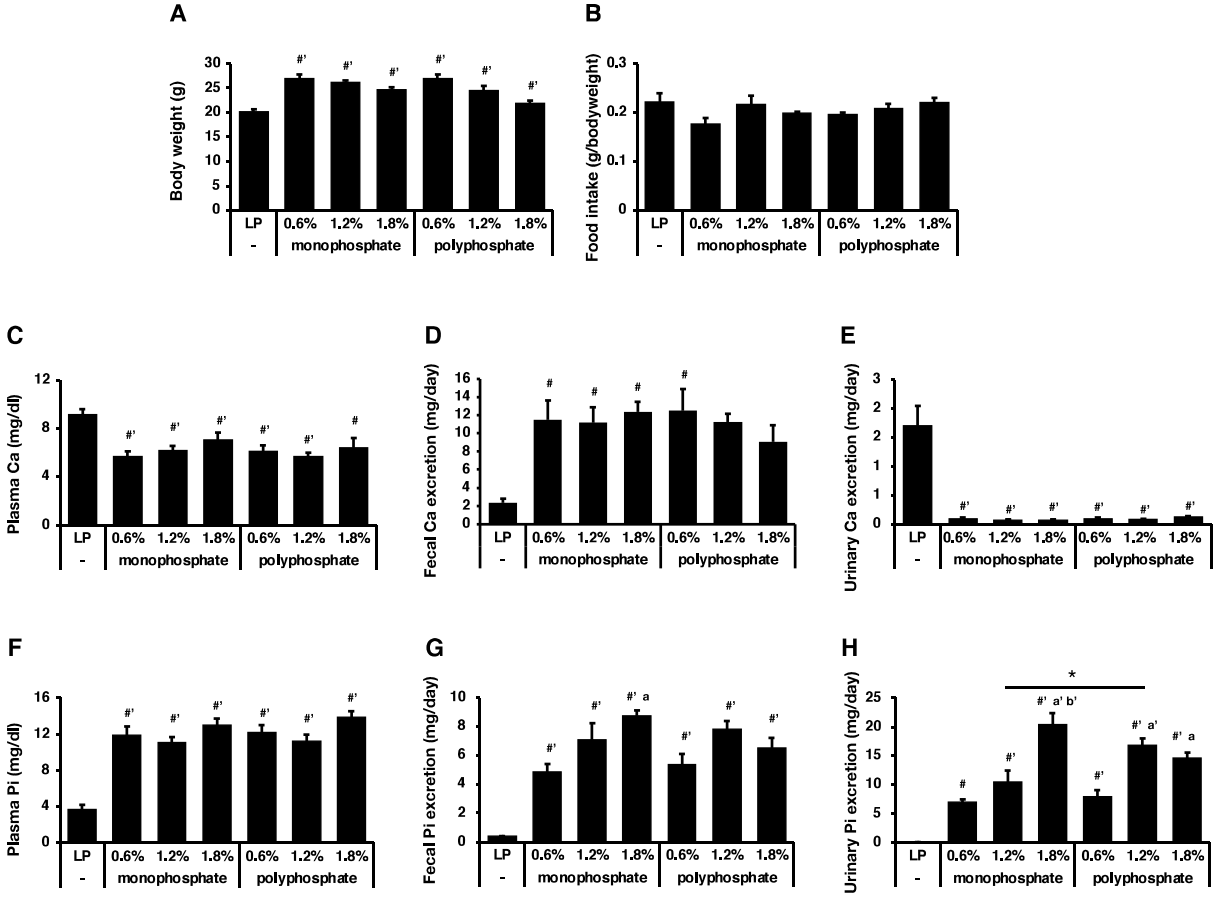


Figure 1. Effects of monophosphate and polyphosphate diets for 5 weeks on body weight, food intake, and biochemical parameters (A) Body weight, (B) food intake, (C) blood calcium concentration, (D) fecal calcium excretion, (E) urinary calcium excretion, (F) plasma Pi concentration, (G) fecal Pi excretion, (H) urinary Pi excretion. Data are presented as mean ± SE, n = 3–4. $^{\#}p < 0.05$, $^{\#'}p < 0.01$ (versus LP), $^{a}p < 0.05$, $^{a'}p < 0.01$ (versus 0.6% diet, same Pi salt), $^{b'}p < 0.01$ (versus 1.2% diet, same Pi salt), $^{*}p < 0.05$

dilatation and tubular epithelial thinning, whereas the proximal straight tubules demonstrated tubular degradation and interstitial fibrosis (Figure 2F-ii, -iv). At the medullary-cortical interface, the 1.8% PPi group showed more severe tubular dilatation and interstitial fibrosis than the 1.8% MPi group (Figure 2F-i, iv), while the overall PPi group showed more severe calcification compared to the MPi group (Figure 2G-ii, -iii, v-, -vi).

*Effects of monophosphate and polyphosphate diets on phosphaturic hormone levels and renal NaPi transporter expression*

In the MPi group, plasma PTH levels were either significantly increased or had an increasing tendency with increasing Pi content. In the PPi group, only the 1.8% PPi diet had significantly increased plasma PTH levels compared to the LP diet (Figure 3A). Regarding serum FGF23 levels, the 1.8% MPi and 1.8% PPi diets were significantly elevated compared to the 0.6% and 1.2% Pi groups, respectively. In particular, the 1.2% and 1.8% PPi diets significantly induced serum FGF23 levels compared to the same level of MPi diets (Figure 3B).

Afterwards, we observed the expression of renal NaPi transporters in the kidneys of mice fed with the 0.6% and 1.2% MPi or PPi diet (Figure 4). No variations in Npt2a and Npt2c mRNA expression levels were observed in all groups (Figure 4A). In both MPi and PPi groups, mice in the 1.2% diet group, compared to the 0.6% diet group, had either a significant reduction or a tendency for reduction in Npt2a and Npt2c protein expression in

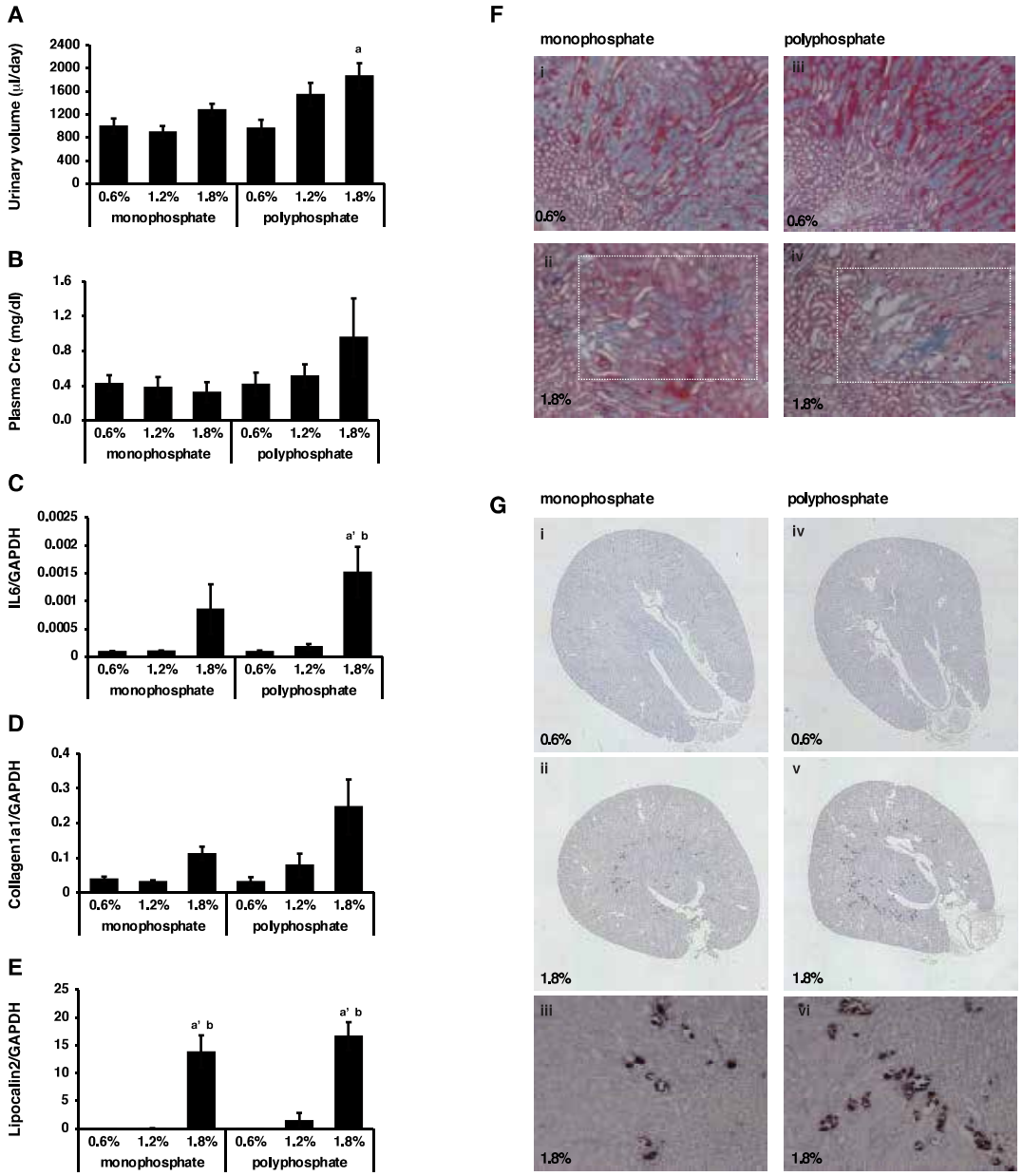


Figure 2. Effects of monophosphate and polyphosphate diets for 5 weeks on renal inflammation, fibrosis, and calcification
(A) Urinary volume, (B) plasma Cre, Real-time PCR (C) renal IL-6, (D) renal collagen1a1, (E) renal Lipocalin 2. GAPDH was used as an internal control. $^{a}p < 0.05$, $^{a'}p < 0.01$ (versus 0.6% diet, same Pi salt), $^{b}p < 0.05$ (versus 1.2% diet, same Pi salt). Data are presented as means ± SE, n = 3–4. (F) Masson's trichrome staining. Monophosphate diet (i, ii). Polyphosphate diet (iii, iv). Original magnification : x100. Areas of severe tubular dilatation and interstitial fibers are indicated by dotted lines. (G) Von Kossa staining. Monophosphate diet (i-iii). Polyphosphate diet (iv-vi). Original magnification : x40 (i, ii, iv, and v), x100 (iii, vi).

the kidneys (Figure 4B). Furthermore, protein expression levels were more reduced in the PPi group compared to the MPi group.

*Effects of monophosphate and polyphosphate diets on the intestinal phosphate transporter and intestinal alkaline phosphatase*

We examined the expression of Pi homeostasis-related molecules in the intestines of mice fed with MPi and PPi diets. Figure 5 shows the results 1 week into the LP, 1.2% MPi, and 1.2% PPi diets. Both 1.2% MPi and 1.2% PPi diets significantly suppressed the expression of Npt2b protein, an intestinal NaPi transporter (Figure 5A). Interestingly, the expression and activity of intestinal alkaline phosphatase protein were significantly suppressed only in the MPi group and not in the LP and PPi groups (Figure 5B, and 5C).

## DISCUSSION

In the present study, we investigated the biological response of MPi and PPi salt in mice. Mice fed with the high Pi diet (1.2% or 1.8% Pi salt) had increased urinary and fecal Pi excretion, blood PTH concentration, and blood FGF23 concentration compared to the control diet group (0.6% Pi salt). The kidneys of mice under a high-Pi diet with MPi or PPi as the source of phosphorus showed elevations in marks of inflammatory (IL-6), fibrosis (collagen1a1), and tubular inflammation (Lipocalin-2), with no differences between the Pi sources. However, the kidneys of mice under a high-Pi diet using PPi as the phosphorus source, compared to MPi, were more severely damaged as described previously in rats (19, 20). In this study, more severe tubular

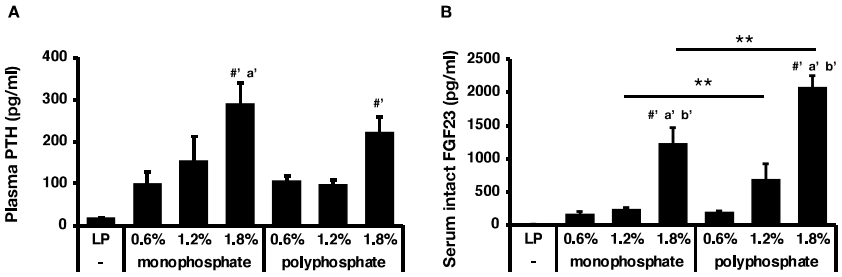


Figure 3. Effects of monophosphate and polyphosphate diets for 5 weeks on the phosphaturic hormones (A) Plasma PTH, (B) serum FGF23. Data are presented as means ± SE, n = 3–4. $^{\#}p < 0.01$ (versus LP), $^{a'}p < 0.01$ (versus 0.6% same phosphate salt), $^{b'}p < 0.01$ (versus 1.2% same phosphate salt), $^{**}p < 0.01$

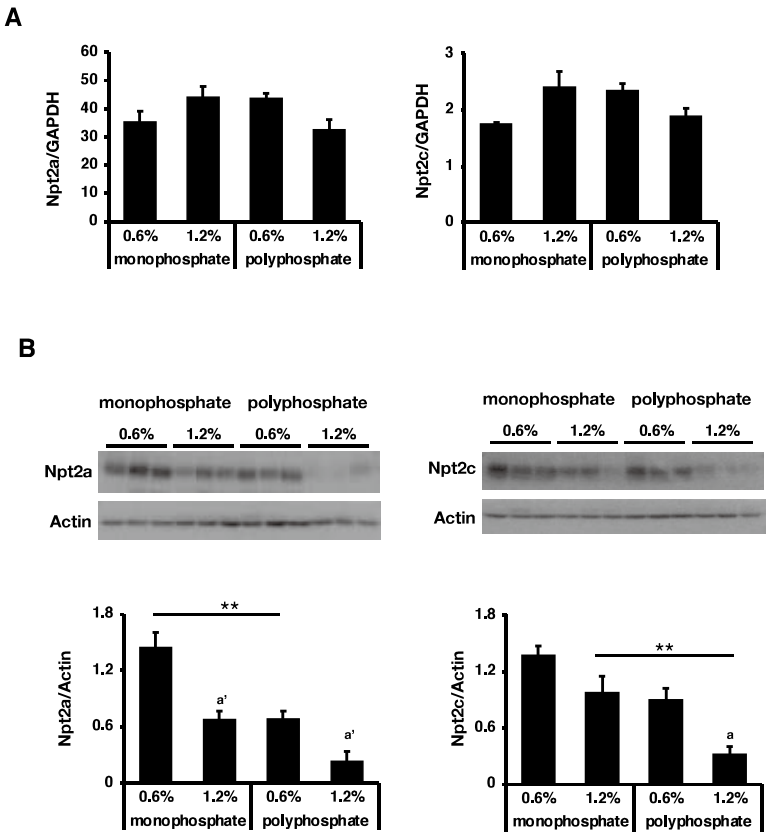


Figure 4. Effects of monophosphate and polyphosphate diets for 5 weeks on renal NaPi transporters expression (A) Real-time PCR renal Npt2a and Npt2c. GAPDH was used as an internal control. (B) Western blotting analyses. Each lane was loaded with 20 μg of renal BBMVs. Actin was used as an internal control. Data are presented as means ± SE, n = 3–4. $^{a}p < 0.05$, $^{a'}p < 0.01$ (versus 0.6% same phosphate salt), $^{**'}p < 0.01$.

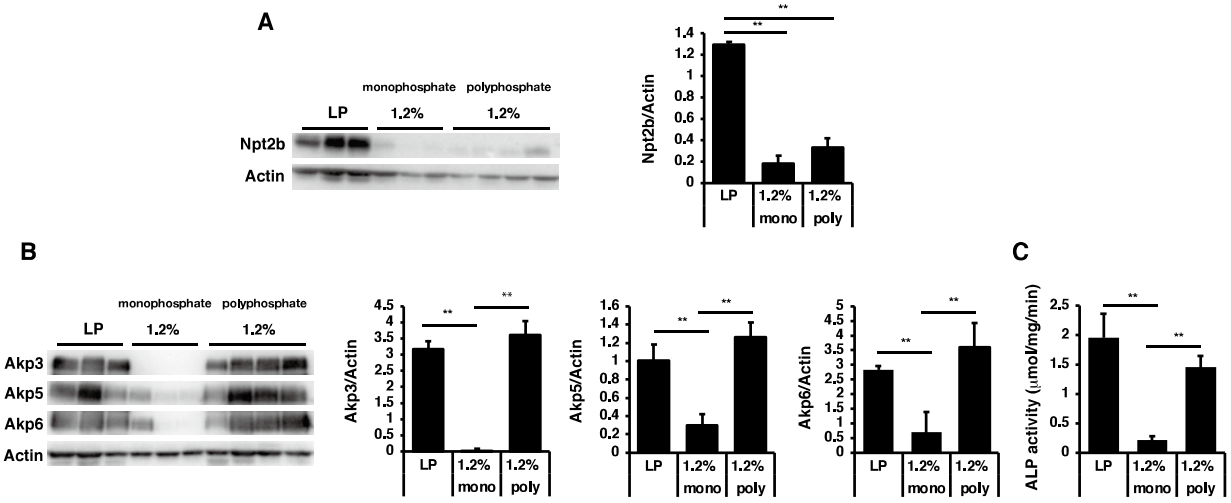


Figure 5. Effects of monophosphate and polyphosphate diets on intestinal NaPi transporter and intestinal alkaline phosphatase protein expression

Western blotting analyses. Each lane was loaded with 20 μg of intestinal BBMVs of mice fed with the monophosphate or polyphosphate diet for 1 week. Actin was used as an internal control. (A) Npt2b protein expression in the distal segment, (B) intestinal alkaline phosphatase (IAP) protein (Akp3, akp5, and Akp6) expression in the proximal segment. Data are presented as means ± SE, n = 3–4. (C) IAP activity in the proximal segment. Data are presented as means ± SE, n = 3–4. $^{**}p < 0.01$

dilatation, interstitial fibrosis, and severe calcification at the medullary-cortical interface were observed in the 1.8% PPi group compared to the 1.8% MPi group. However, the details of this effect are yet to be elucidated. Therefore, in this study, we focused on the effects of MPi and PPi diets on the PTH and FGF23 secretion and Pi transporter expression in the kidney and intestine.

There was no difference in plasma Pi and PTH levels in mice fed with the high Pi diet with PPi as the phosphorus source compared to MPi, but there was a significant increase in serum FGF23 levels. FGF23 is produced mainly in osteocytes and binds to the α-Klotho-FGF receptor complex to promote Pi excretion by suppressing Npt2a and Npt2c expression in the renal proximal tubules (2-4). In fact, the levels of Npt2a and Npt2c protein expression were more suppressed in the PPi group. This phosphaturia by FGF23 increases urinary Pi concentrations and increases the risk of CaPi formation in the proximal tubular fluid. It has been reported that Pi excretion per nephron, but not plasma Pi level, correlates with histological change scores reflecting the severity of tubular damage and interstitial fibrosis (28, 29). In the 1.2% high-Pi diet group, phosphaturia was significantly elevated when PPi was used as a phosphorus source versus MPi. High FGF23, which increases phosphate excretion per nephron, may induce tubular damage and renal fibrosis by promoting CaPi formation in the tubular fluid. Therefore, the renal injury in mice fed a high-phosphorus diet using PPi as the phosphorus source may can be attributed to the enhanced secretion of FGF23. Since renal injury also leads to the induction of FGF23, a vicious cycle may be induced by a high-phosphorus diet with PPi as the phosphorus source. FGF23 promotes phosphaturia and suppresses vitamin D synthesis, dependent on α-Klotho. In contrast, FGF23 has been reported to promote left ventricular hypertrophy (LVH) in the heart and the production of inflammatory cytokines in the liver, suppress mineralization in bone, and inhibit the production of erythropoietin in the bone marrow in α-Klotho-independent manner (30). Thus, high serum FGF23 levels in the high-phosphorus PPi diet group may promote systemic inflammation more strongly than the MPi equivalent.

Finally, we examined the effects of test diets with MPi or PPi as the phosphorus source on the intestinal tract. Pi absorption is thought to be mediated by transcellular transport (e.g., using the sodium-dependent Pi transporter Npt2b) and by passive transport via paracellular transport (3, 4). The molecular mechanism of paracellular-mediated Pi transport is not clear. The substrate of Npt2b is MPi, and it is thought that various forms of phosphorus in the diet are digested and absorbed in the form of MPi (13). A similar trend was seen in intestinal Npt2b protein expression when comparing MPi and PPi diets, but intestinal alkaline phosphatase protein expression was suppressed only in the high-phosphorus MPi diet. We have previously reported that intestinal alkaline phosphatase (which is suppressed by a high phosphorus diet and increased by a low phosphorus diet) and Npt2b are both involved in intestinal Pi transport (22). The mechanism behind this is unknown, but our findings suggest that it may recognize MPi or PPi in the intestine. Further studies are needed to elucidate the factors and mechanisms involved in the recognition of phosphorus forms in the intestine and the regulation of FGF23 secretion.

In conclusion, PPi, which has a more severe effect on renal damage than MPi, promotes FGF23 secretion. Excess FGF23 may be more involved in inflammation, fibrosis, and calcification in the kidney.

## CONFLICT OF INTEREST

None

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## AUTHOR CONTRIBUTION

Conceived of and designed the research ; S.S. and H.S. Performed the experiments ; S.S., M.K., K.T., M.U., K.K., A.K., M.M., Y.H., Y.H., SH.S., Y.S., I.K., and H.S. Analyzed the data ; S.S. and H.S. Prepared Figures : S.S. and H.S. Drafted the manuscript ; S.S., and H.S. Reviewed and approved manuscript ; K.M., and H.S.

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