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Original Article

Impact of altered dietary calcium–phosphorus ratio caused by high-phosphorus diets in a rat chronic kidney disease (CKD) model created by partial ligation of the renal arteries

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Abstract: This study aimed to establish a rat chronic kidney disease (CKD) model by studying the effects of a high-phosphorus diet in rats that had undergone partial ligation of the renal arteries (RL). Separate groups of 10-week-old male Slc:Sprague-Dawley rats underwent RL and were fed diets with varying phosphorous levels for a period of 48 days. A marked suppression of body weight gain necessitating humane euthanization occurred on day 28 in rats that had undergone RL and were given high-phosphorus feed. By contrast, the group of intact animals on a high-phosphorus feed exhibited a slightly decreased body weight gain from day 21 and survived until scheduled euthanization. In rats with RL, hematological, blood biochemical, and histopathological analyses demonstrated the presence of CKD-like conditions, particularly in the group that were fed a high-phosphorus diet. Hyperphosphatemia and hypocalcemia were induced by a high-phosphorus diet in both the RL and intact groups, both of which had high levels of FGF23 and parathyroid hormone in the blood. Rats with RL on a high-phosphorus diet showed decreased hematopoiesis by the hematopoietic cell area being narrower in the medullary cavity, proliferation of mesenchymal cells and osteoblasts/osteoclasts, and expansion of the osteoid area, a furthermore generalized vascular lesions, such as calcification, were observed. These findings demonstrate that the partial ligation of the renal arteries combined with a calcium–phosphorus imbalance induced by a high-phosphorus diet serves as an animal model for CKD-like conditions accompanied by bone lesions, helping to elucidate this clinical condition and its underlying molecular mechanisms. (DOI: 10.1293/tox.2019-0086; J Toxicol Pathol 2020; 33: 77–86)

Key words: diet food, calcium, phosphate, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), rats

Introduction

In patients with chronic kidney disease (CKD), a decline in renal function results in a significant reduction in urine output. This renders the body incapable of excreting water and waste products, which may in turn induce various diseases. One of the most common diseases is hyperphosphatemia, which is caused by defective phosphorus excretion. Patients with CKD are typically treated with dialysis to remove water and waste products from their bodies. However, the amount of phosphorus that can be eliminated in one dialysis session is insufficient, and often causes the blood phosphorus levels to increase. This commonly occurs when a patient consumes a large amount of phosphorus in their diet in excess of what can be removed through dialysis. If left unchecked, phosphorus accumulates in the body, which induces hyperphosphatemia. If hyperphosphatemia is allowed to persist, ectopic calcification and osteoporosis

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may be induced[1]. These clinical conditions have been shown to play a role in the increased risk of mortality in the form of vascular calcification. The concept of "CKD-mineral and bone disorder (CKD-MBD)" was introduced in *Kidney Disease: Improving Global Outcomes (KDIGO)*[2].

Under normal conditions, humans consume 1,200 mg/day of phosphorus. The body absorbs 950 mg of this, 29% of which is stored in the bone and less than 1% in the blood. The remaining 70% of absorbed phosphorus exists in cells in a form readily available for exchange. Phosphorus is excreted from the body at a rate of 150 mg/day in the stool and 800 mg/day in the urine[3]. The levels of phosphorus in the blood are regulated by two phosphaturic factors: parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), which are released from the bone[4, 5]. PTH works on parathyroid hormone receptor 1 (PTHR1) to reduce the amount of phosphate transporter, thus promoting phosphaturesis[5]. FGF23 binds to klotho and FGF receptor 1 (FGFR1), promoting the excretion of phosphorus[4] and inhibiting the renal synthesis of 1,25-$(OH)_2D_3$, which in turn reduces the level of 1,25-$(OH)_2D_3$ in the blood and suppresses intestinal phosphorus absorption[4]. In a healthy individual, the phosphaturic factors in the parathyroid and bone (i.e., PTH and FGF23, respectively) act in response to a high-phosphorous load to eliminate excess phosphorus from the body. In an individual with CKD, especially in the advanced stages, the levels of PTH and phosphorus in the blood are increased, causing the levels of activated vitamin D to decline, while promoting the synthesis and secretion of PTH due to hyperphosphatemia, which can eventually induce secondary hyperparathyroidism[5]. Although FGF23 acts to inhibit the parathyroid to synthesize or excrete PTH[6, 7], when CKD is in the advanced stages, the expressions of klotho and FGFR1 in the parathyroid are decreased and its resistance to the inhibitory action of FGF23 is induced[8]. In addition, as CKD progresses, more calcium is released from the bone, causing a storage of calcium and phosphorus to form in the blood and other soft tissues, which could result in the development of ectopic calcification[9]. In short, abnormal levels of PTH, calcium, or phosphorus in patients with CKD may result in the development of metabolic bone disease.

In patients with CKD, the metabolism of calcium and phosphorus plays an important role in determining the severity and prognosis of CKD, as well as the patient's quality of life. Because the dietary content of calcium and phosphorus, as well as their ratio, have a large impact on the metabolism of calcium and phosphorus, these factors are also considered to be key to CKD. However, experimental studies on their role in CKD are limited. Accordingly, the current study established a rat model where the renal arteries are partially ligated in an effort to establish CKD accompanied by hyperphosphatemia. The partial ligation of the renal arteries was performed by ligating three of the four renal artery branches, leaving one intact, in each of the two kidneys. This allowed for a decreased number of functional glomeruli while applying stress on the kidneys. This procedure also increased blood pressure and intraglomerular pressure, which could cause kidney disease, with symptoms including damage to the glomerular basement membrane and protein leakage, eventually resulting in CKD accompanied by interstitial fibrosis. This model was used to analyze the impact of an altered dietary calcium–phosphorus ratio caused by a high-phosphorus diet.

Materials and Methods

Animals and housing conditions

Animals: Male Slc:Sprague-Dawley rats were supplied by Japan SLC Inc. (Shizuoka, Japan). and housed individually in polycarbonate cages with stainless steel wire lids (W25 × D40 × H18 cm) throughout the study period. The animals were kept in a room maintained at 21.0–25.0°C and 40.0–70.0% humidity, in a 12/12 h light/dark cycle, and given *ad libitum* access to tap water for 48 days.

This study was conducted in compliance with the Act on Welfare and Management of Animals and the Asahi Kasei Medical Animal Experimentation Policy in accordance with the study protocol reviewed by the animal welfare committee of the laboratory.

Diets: The animals were given *ad libitum* access to solid feed from PMI Nutrition International, LLC for a period of 48 days. Each type of feed was identified in terms of the ratio of phosphate to calcium: "1/2 forage" (5WJX [Ca: 0.61%; P: 0.30%]); "1 forage" (5755 Basal [Ca: 0.61%; P: 0.57%]); "2 forage" (5WJY [Ca: 0.61%; P: 1.20%]); and "4 forage" (5WJZ [Ca: 0.61%; P: 2.40%]). Animals in the control group were given "1/2 forage" (5WJX [Ca: 0.61%; P: 0.30%]).

Experimental design

A total of 30 rats were purchased at 10 weeks of age. Of these, 20 rats underwent partial ligation of the renal arteries (RL) prior to the start of the study, while the remaining 10 were left untreated. Prior to purchase partial ligation of the renal artery was performed under anesthesia on rats at eight weeks of age by ligating three of the four branches of the renal artery to the left kidney. After ligation, the kidney was returned to the abdominal cavity and the flank incision was sutured. At nine weeks of age, the rats underwent the same procedure on the renal artery to the right kidney. These treatments were provided by Japan SLC Inc.

The experiment was conducted in six study groups, each containing five animals, as shown in Table 1. The study period was 49 days, at the end of which the animals were humanely euthanized and necropsy was performed.

General conditions

The rats were observed for general condition on a daily basis between the start of the feeding study (hereafter baseline) and the day of necropsy.

Body weight and food/water consumption

Body weight, food consumption, and water consumption were measured once per week between the baseline and the day of necropsy.

Table 1. Study Group Structure and Feed Content (Calcium and Phosphorus)

Group	RL	Diet food condition			Number of animals
		Calcium (%)	Phosphorus (%)	Ca/P ratio	
Control	n.d.	0.61	0.3	1/2	5
N-2.4	n.d.	0.61	2.4	4	5
RL-0.3	Treated	0.61	0.3	1/2	5
RL-0.6	Treated	0.61	0.57	1	5
RL-1.2	Treated	0.61	1.2	2	5
RL-2.4	Treated	0.61	2.4	4	5

n.d.: Not done. RL: partial ligation of the renal arteries.

Urinalysis

Urine was collected from rats placed in metabolic cages during a 24-h period preceding each of the days of blood collection for blood biochemistry-1 (see below). The urinary volume was measured. Additional urinalysis parameters consisted of creatinine, urea nitrogen, inorganic phosphorus, calcium, urinary microalbumin, and N-acetylglucosaminidase, which were analyzed using a Biolis 24i Premium, a compact automatic analyzer system for clinical examinations marketed by Tokyo Boeki Medisys (Tokyo, Japan).

Hematology

Upon necropsy, blood samples were collected from the abdominal aorta of rats and analyzed for red blood cells, white blood cells, platelets, hemoglobin levels, hematocrit levels, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration using the Sysmex automated hematology analyzer XT-2000iV.

Blood biochemistry-1

Samples were prepared from venous blood collected 1 day before the baseline and 7, 14, 21, 28, 35, 42, and 49 days after the baseline. The blood was collected from the abdominal aorta during necropsy. The prepared samples were analyzed for creatinine, blood urea nitrogen (BUN), electrolytes (sodium, potassium, chlorine, phosphorus, calcium, and magnesium) using the Biolis 24i Premium. The samples prepared during necropsy were analyzed for aspartic acid and alanine aminotransferases, alkaline phosphatase (ALP), γ-glutamyltransferase, amylase, total cholesterol, triacylglycerol, glucose, free fatty acids, creatinine kinase, total protein, albumin, bilirubin, and electrolyte (magnesium).

Blood biochemistry-2

The serum samples collected during necropsy were analyzed for levels of parathyroid hormone (PTH) and FGF23.

Histopathology

During necropsy, the following organs were sampled, weighed, and fixed in 10% (vol) neutral buffered formalin solution: liver, kidneys, adrenal glands, stomach, duodenum, jejunum, ileum, colon, rectum, cecum, esophagus, pancreas, spleen, heart, lungs, trachea, thoracic aorta, testes, epididymis, prostate gland, bladder, thymus gland, mesenteric lymphatics, thyroid glands, parathyroid glands, femur, muscle, brain (cerebellum and cerebrum), and pituitary gland. Hematoxylin and eosin (H&E)-stained samples were prepared according to the standard procedures. All samples were examined microscopically.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. $P<0.05$ was considered significant.

All statistical analyses were conducted using Bell Curve for Excel version 3.20 (Social Survey Research Information).

Results

Body weights were slightly lower in all of the partial ligation groups compared to those in the control group, both before and at the baseline. The body weight in the RL-2.4% group continued to decline during the study, reaching levels warranting euthanasia on day 28 from the perspective of animal ethics (i.e., a body weight ≤ 80% of that on day 0); these animals were therefore euthanized before scheduled necropsy. The body weight tended to be low in the N-2.4% group as a result of decreased body weight gain, although the change was not significant. No changes were observed in any of the other groups (Fig. 1).

Water intake, urine output, and food consumption

Compared to the control group, the N-2.4%, RL-1.2%, and RL-2.4% groups almost consistently showed high levels of urine output (Fig. 2) throughout the duration of the study. Similar trends were observed in terms of the water intake, while only slight changes were observed regarding food consumption.

Urinalysis

The levels of phosphorus in the urine were high from day 7 until the animals' emergency or scheduled necropsy in the N-2.4%, RL-1.2%, and RL-2.4% groups, particularly in the N-2.4% and RL-2.4% groups (Fig. 3A). The levels of calcium in the urine were high in the final measurement

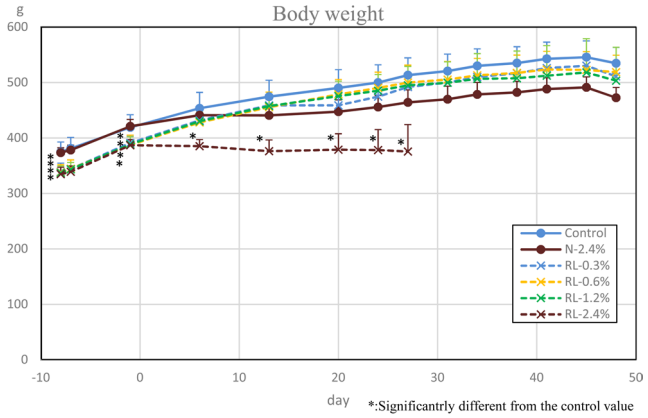

Fig. 1. Body weights.

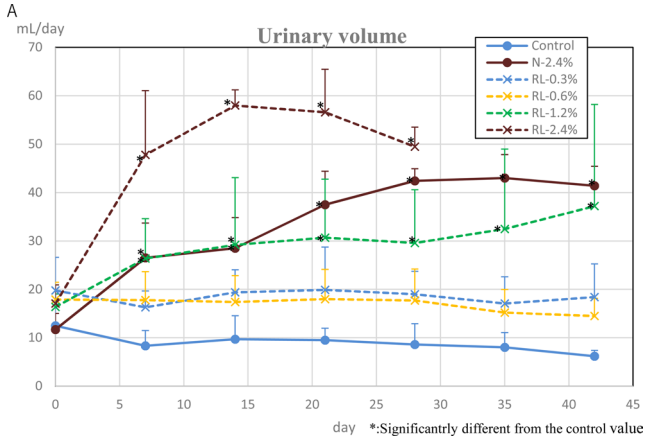

Fig. 2. Urinary volumes.

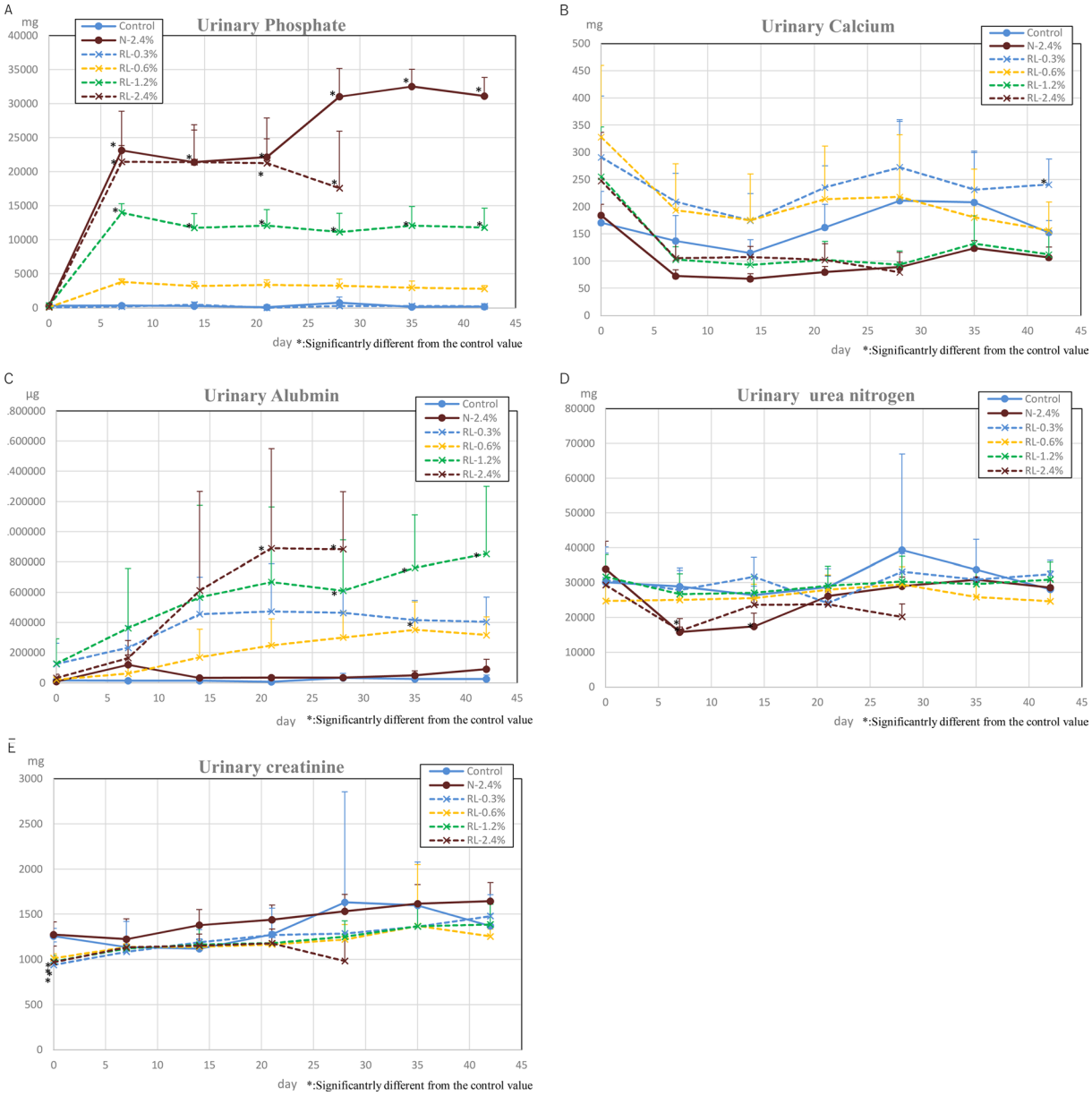

Fig. 3. Urinalysis: level of phosphate, calcium, albumin, and urea nitrogen.

of the RL-0.3% group, while they tended to be low in the N-2.4%, RL-1.2%, and RL-2.4% groups on and after day 21, although the changes were not significant (Fig. 3B). The urine albumin levels were high in the RL-1.2% and RL-2.4% groups, and tended to be high in all the other groups, except for the control and N-2.4% groups (Fig. 3C). The urinary urea nitrogen levels were high in the N-2.4% group on days 7 and 14, and in the RL-2.4% group on day 7 (Fig. 3D). The creatinine levels were smaller at the baseline in all of the groups of animals that had undergone RL (Fig. 3E). No changes were observed in any of the other test items.

Hematology

The red blood cell counts (Fig. 4), hemoglobin levels, and hematocrit levels were low in the RL-2.4% group and tended to be low in the N-2.4% group, although the differences were not significant. The white blood cell counts were high in the RL-2.4% group. No changes were observed in any of the other test parameters or in the other groups.

Blood biochemistry-1

The levels of phosphorus in the blood were markedly high in the RL-2.4% group from day 7 until emergency necropsy, while the N-2.4% and RL-1.2% groups occasionally showed high levels (Fig. 5A). The levels of calcium in the blood were low in the RL-2.4% group and tended to be low in N-2.4% group, although the difference was not significant (Fig. 5B). The BUN levels were high at the start of the feeding study in the groups of animals that had undergone partial ligation of the renal artery, while the N-2.4%,

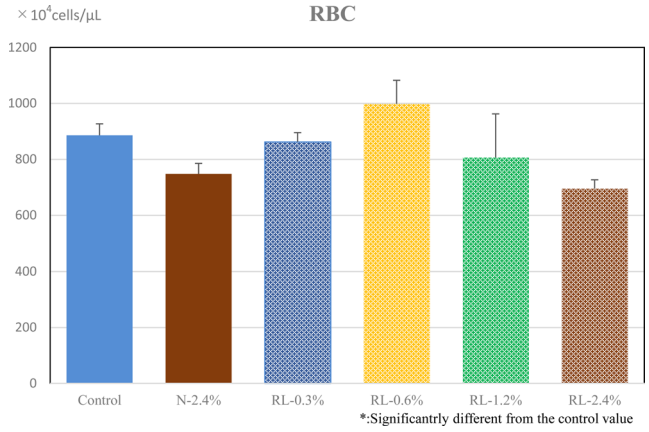

Fig. 4. Hematology: level of red blood cells.

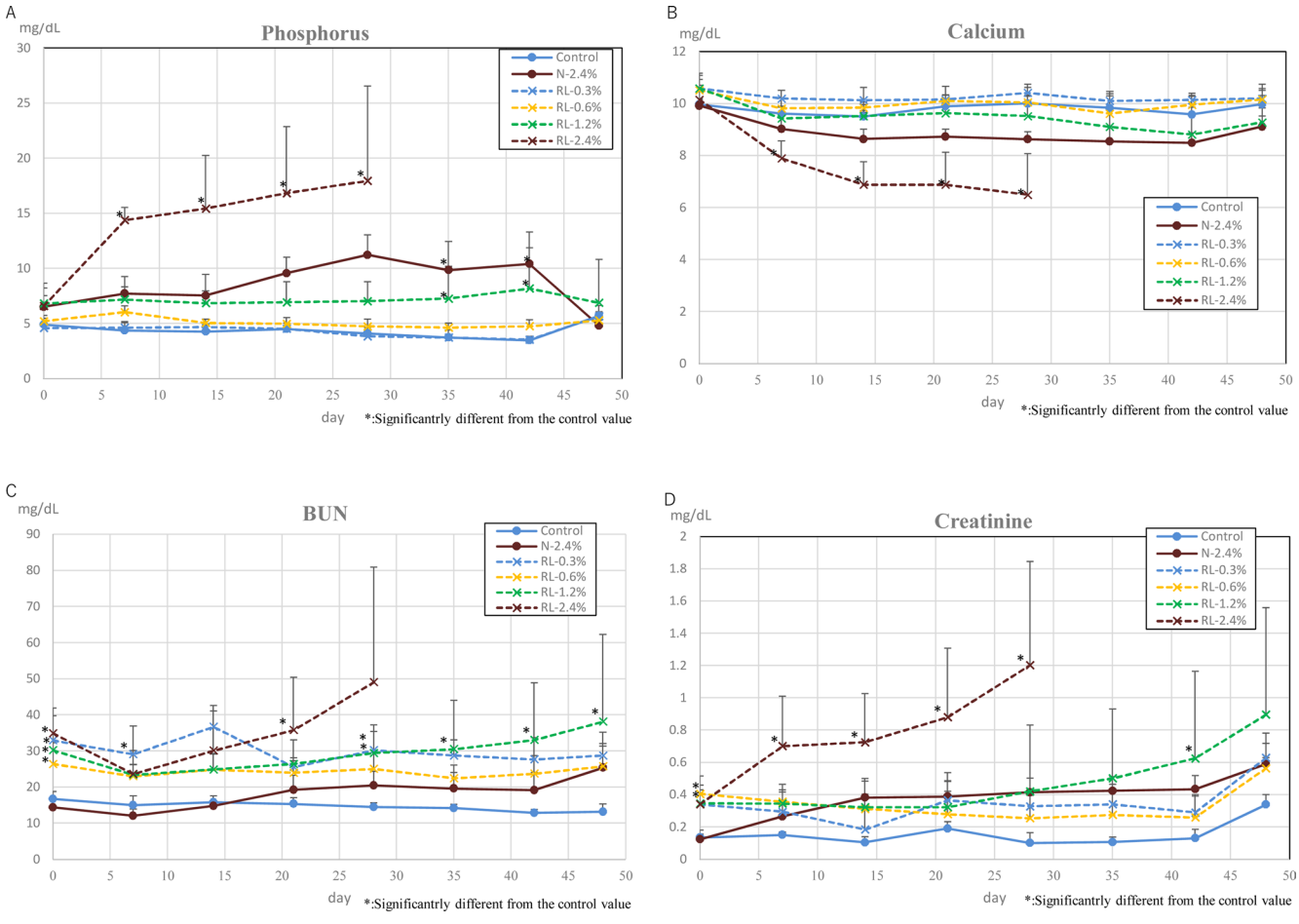

Fig. 5. Blood biochemistry: level of phosphate, calcium, blood urea nitrogen, and creatinine.

L-0.3%, and RL-2.4% groups occasionally showed high levels (Fig. 5C). The creatinine levels were also high at the start of feeding in the groups of animals that had undergone RL, while the N-2.4% group continued to show high levels thereafter (Fig. 5D). The ALP levels reported are those at the time of necropsy; the levels tended to be high in the RL-2.4% group, although the difference was not significant (Fig. 6). No changes were observed in any of the other test items or in the other groups.

Blood biochemistry-2

The levels of FGF23 were high in the RL-2.4% group. The levels of FGF23 PTH tended to be high in N-2.4% and RL-1.2% groups, although the differences were not significant (Fig. 7A and B).

Organ weight

The absolute kidney weights (Fig. 8A and B) increased in the N-2.4% and RL-2.4% groups, while the relative weights increased in the RL-2.4% group. No changes were observed in any other organs or in other groups.

Histopathology

In all of the groups of rats that had undergone RL, the bound areas were contracted and replaced by connec-

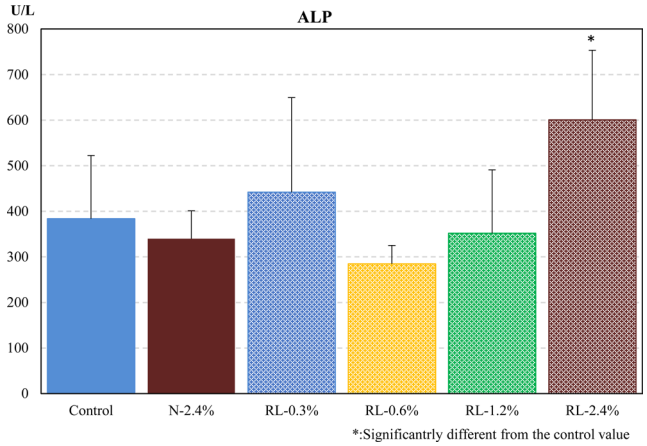

Fig. 6. Blood biochemistry: level of alkaline phosphatase.

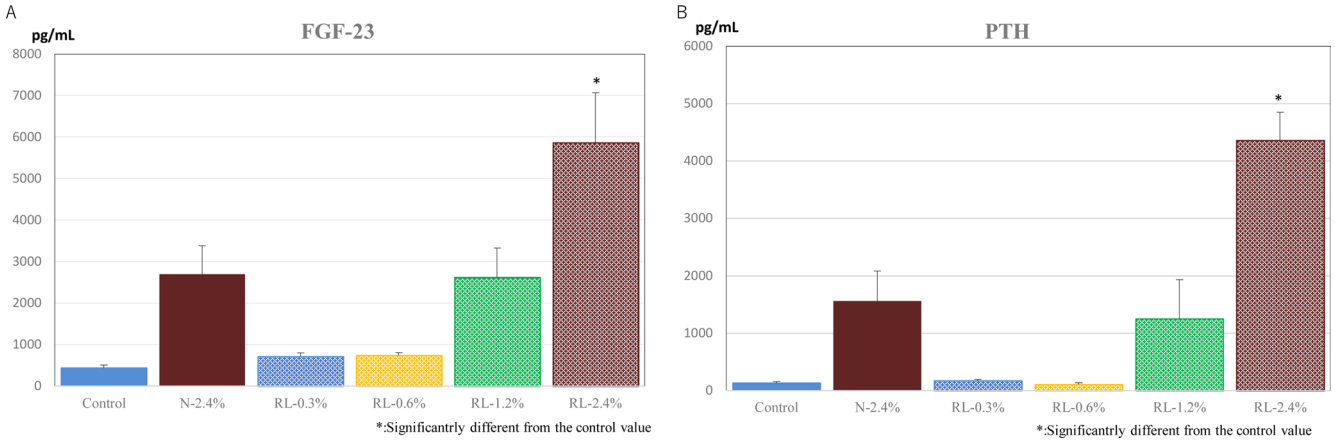

Fig. 7. Blood biochemistry: level of fibroblast growth factor 23 and parathyroid hormone.

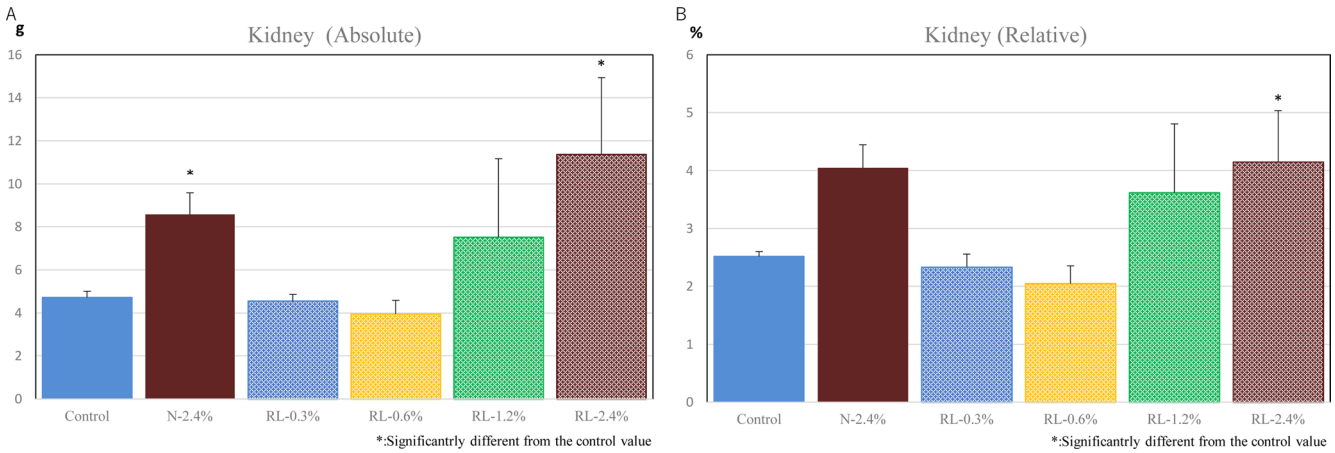

Fig. 8. Absolute and relative kidney weights.

tive tissues, and inflammatory cell infiltration, the degeneration and necrosis of residual nephrons, and calcification were observed. These lesions were diagnosed as being infarct areas caused by ligation (Fig. 9A and B). There were clearly defined boundaries between the ligated areas and normal tissues; in the areas around the boundaries, inflammatory interstitial cell infiltration, urinary casts, expansion of the renal tubular lumen, fibrous thickening of the Bowman's capsule and the renal tubular basement membranes, and the degeneration and regeneration of renal tubular epithelial cells were observed. In the RL-2.4% group, whose phosphorus intake had been further increased, the kidneys were enlarged compared to those in the RL-0.3% group, and expansion of the renal tubular lumen and urinary casts were also observed (Fig. 9C). In addition, inflammatory cell infiltration, fibroblast proliferation, calcification, and the degeneration and regeneration of epithelial cells accompanied by thickening of the basement membranes were observed throughout the entire kidneys (Fig. 9D). In the glomeruli, expansion of the afferent arteriole lumen and glomerular capillaries, enlargement of podocytes, proliferation of epithelial cells of the Bowman's capsule, thickening of the Bowman's capsule wall (Fig. 9E), adhesion of the glomeruli to the Bowman's capsule wall, and swelling of mesangial cells (Fig. 9F) were also observed. These changes were observed to lesser degrees in the RL-1.2% group, and not at all in the RL-0.3% and RL-0.6% groups. In the N-2.4% group, the renal cortex was shown to be basophilic in a wedge-shaped area, and an expansion of the renal tubular lumen was also observed (Fig. 9G). These changes were almost the same as those observed in the RL-2.4% group, although no expansion of the afferent arteriole lumen and glomerular capillaries was observed (Fig. 9H).

In the RL-2.4% group, some of the findings in organs other than the kidneys included a marked enlargement of the parathyroid compared to that in the control group, which was apparent even under low magnification. Under high magnification, the nuclei of the parathyroid parenchymal cells appeared bright, while the chromatins were large and disproportionately concentrated in the cornea, and the granular cell bodies were prominent in the cytoplasm (Fig. 10A and B). The area directly below the growth plate at the distal end of the femur appeared to be clearer, with the trabecular bone area being markedly thicker and the hematopoietic cell area narrower (Fig. 11A). Under a high magnification, a marked proliferation of mesenchymal cells compared to that in the control group was observed. In the trabecular bone, a marked growth of osteoblasts was observed, as well as a large number of osteoclasts (Fig. 11B). In addition, activated osteoblasts formed a line, creating a marked increase in the osteoid area between them and the existing bones (Fig. 11C). Furthermore, calcification was observed in the blood vessels throughout the body, as well as in the gastric glands and cardiac muscle cells.

No notable histopathological changes were observed in any of the other study groups.

Discussion

This study was conducted to establish a rat CKD model by studying the effects of high dietary phosphorus in rats that had undergone partial ligation of the renal arteries.

Intact rats

The present (histo)pathological, hematological, and biochemical results, especially shown in the N-2.4% group, demonstrated that a calcium–phosphorus imbalance caused by a high-phosphorus diet could induce CKD-like conditions. While the impact of phosphorous on the kidneys has long been discussed[10–14], studies focusing on the effects of altered ratios of dietary calcium–phosphorus are limited. In one study focusing on calcium, where dietary calcium levels ranging from 1/2 to 5 times greater than those of phosphorous were administered for a period of 10 weeks, no symptoms indicative of CKD were observed[15]. The results of this study suggest that to create a state in which homeostasis fails and CKD-like conditions appear, it is necessary to substantially increase the dietary phosphorous levels to create an effective calcium–phosphorus imbalance.

Rats that underwent partial ligation of the renal arteries

In the RL-2.4% group, the general condition of the animals deteriorated. These animals showed high levels of FGF23 and PTH in the blood and high urine and plasma marker levels, as well as histopathological abnormalities characteristic of CKD in the parathyroid glands and kidney. The magnitude of CKD-like conditions was apparently more severe in these rats than in those of the N-2.4% group. The partial ligation of the renal arteries in the RL-2.4% group caused the rats' renal function to deteriorate, while their glomeruli showed damage caused by hypertension and the degeneration and regeneration of epithelial cells. The kidneys clearly underwent intense stress under such conditions, which may have been a result of the early induction and promotion of CKD-like conditions caused by an imbalance in the dietary calcium-phosphorus ratio.

Additionally, in the RL-2.4% group, abnormalities were found not only in the kidneys of the rats, but also in the bone. The findings suggest the involvement of hyperparathyroidism as a background factor, which suggests that conditions similar to those of renal osteodystrophy had been induced.

In conclusion, the experimental conditions used in this study, which combined partial ligation of the renal arteries with a calcium–phosphorus imbalance induced by a high-phosphorus diet, were found to effectively serve as an animal model for CKD-like conditions accompanied by bone lesions, induced in a relatively short period of time. These results will help to elucidate the clinical conditions and underlying molecular mechanisms of CKD.

Our findings provide novel insights supporting the supposition that a calcium–phosphorus imbalance caused by excessive phosphorus consumption in the diet may increase

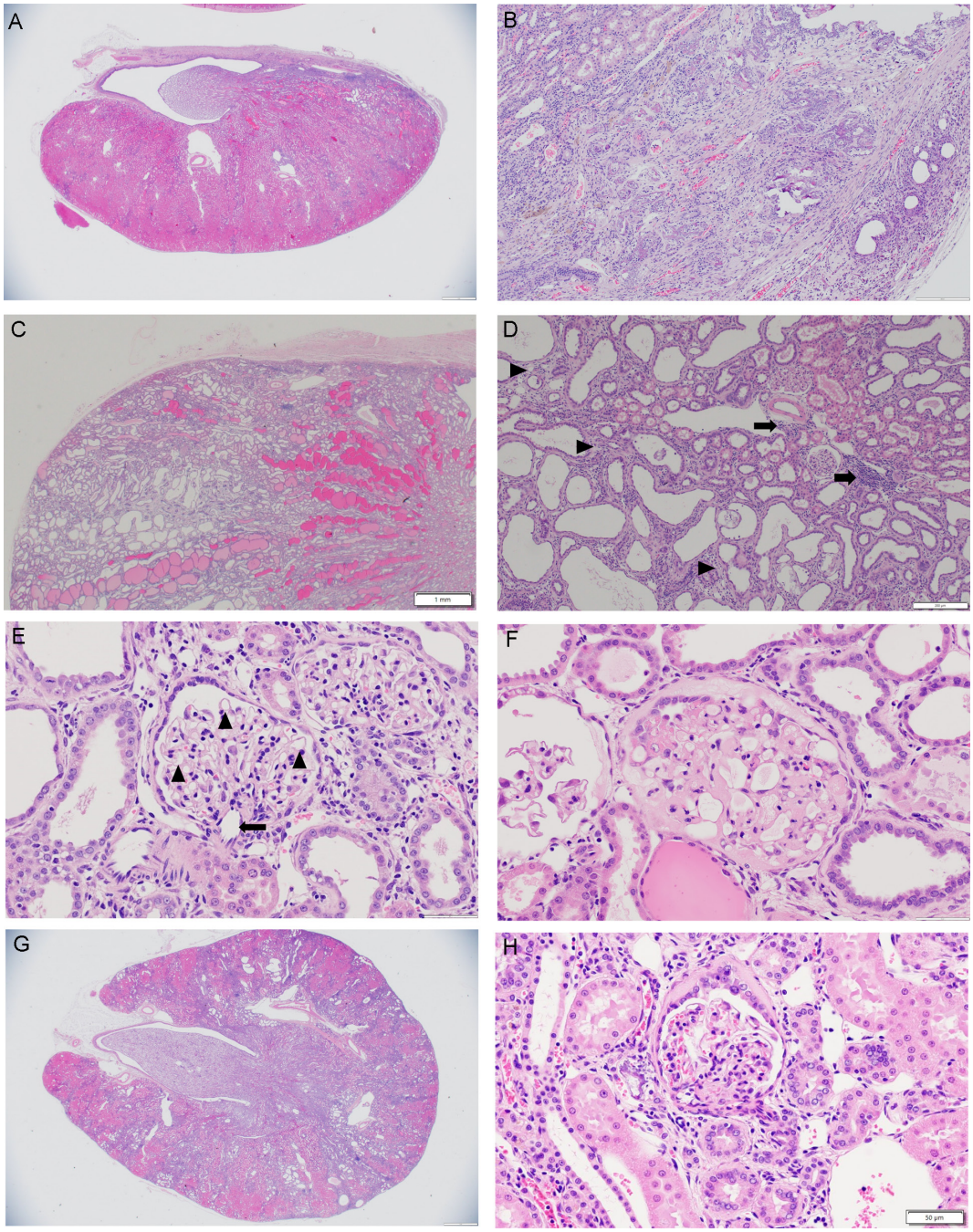

Fig. 9. Histopathology of the kidney. A. RL-0.3%. Partial ligation of the renal artery caused the kidney to form a distorted shape. Bar is 1 mm. B. RL-0.3%, under high magnification. In the infarct areas, inflammatory interstitial cell infiltration, fibrous thickening of the Bowman's capsule and the renal tubular basement membranes, renal tubular epithelial cells, degeneration and regeneration of renal tubular epithelial cells, and calcification, among others, were observed. Bar is 200 µm. C. RL-2.4%. The kidneys were enlarged. An expansion of the renal tubular lumen and urinary cast were also observed. Bar is 1 mm. D. RL-2.4%, under high magnification. In the non-infarct areas, inflammatory cell infiltration (arrows), fibroblast proliferation (arrow heads), calcification, and the degeneration and regeneration of epithelial cells accompanied by the thickening of the basement membranes, among others, were observed. Bar is 200 µm. E. RL-2.4%, under high magnification. In the non-infarct areas, expansion of the afferent arteriole lumen (arrows) and glomerular capillaries (arrow heads), enlargement of the podocytes, the proliferation of epithelial cells of the Bowman's capsule and the thickening of the Bowman's capsule wall were observed in the glomeruli. Bar is 50 µm. F. RL-2.4%, under high magnification. Expansion of the glomerular capillaries, hyaline droplet deposition in podocytes, thickening of the Bowman's capsule wall and its adhesion to the glomeruli, and swelling of mesangial cells, among others, were observed in the non-infarct areas. Bar is 50 µm. G. N-2.4%. The renal cortex was shown to be basophilic in a wedge-shaped area. An expansion of the renal tubular lumen was also observed. Bar is 1 mm. H. N-2.4%. Similar changes to those in the RL-2.4% group were observed, although no expansion of the afferent arteriole lumen or glomerular capillaries was observed in the glomeruli. Bar is 50 µm.

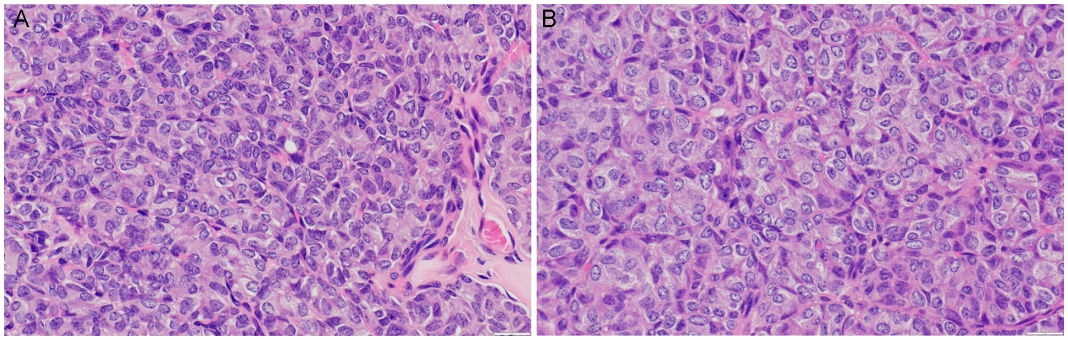

Fig. 10. Histopathology of the parathyroid. A. Control. The parathyroid looks normal. Bar is 20 µm. B. RL-2.4%. The nuclei of the parathyroid parenchymal cells appeared bright, while the chromatin was large and disproportionately concentrated in the cornea. Granular cell bodies were prominent in the cytoplasm. Bar is 20 µm.

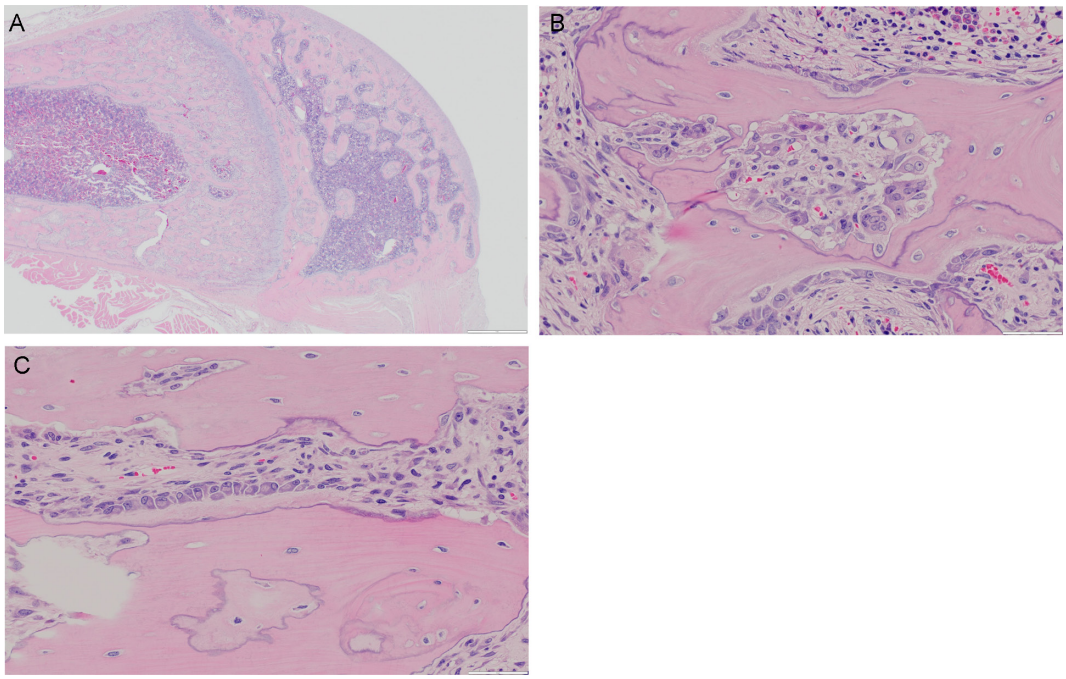

Fig. 11. Histopathology of the bone. A. RL-2.4%. The area directly below the growth plate at the distal end of the femur appeared to be clearer, while the trabecular bone area was markedly thicker and the hematopoietic cell area was narrower. Bar is 1 mm. B. RL-2.4%. The proliferation of mesenchymal cells was marked. In the trabecular bone, a marked growth of osteoblasts was observed, as well as a large number of osteoclasts. Bar is 50 µm. C. RL-2.4%. The activated osteoblasts formed a line, creating a marked increase in the osteoid area between these cells and the existing bone. Bar is 50 µm.

the risk of CKD in humans. The results presented here also indicate that controlling the calcium and phosphorous intake via the diet in patients with early-stage kidney disease may help to delay progression into chronic renal failure, thereby reducing the number of new dialysis patients. These results may also help to improve our understanding of the mechanisms of CKD-MBD and establish a means by which to control this disease.

Disclosure of Potential Conflicts of Interest: The authors declare that there is no conflict of interest.

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