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Impaired Bone Formation with a High-Protein Diet in Rats with Adriamycin-Induced Nephrotic Syndrome

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Key Words
Nephrotic syndrome · High-protein diet · Proteinuria · Bone mineral density · Calcium

Abstract
Background/Aim: The purpose of the study was to investigate the effects of a high-protein (HP) diet on bone metabolism in rats with adriamycin (ADR)-induced nephrotic syndrome. ***Methods:*** Nephrotic syndrome was established by weekly injections of ADR (2 mg/kg, i.p.) for 6 weeks. After a final injection, we confirmed that nephrotic syndrome had developed. Then, the rats were divided into two groups for the dietary treatments, namely the HP diet (30% of calories from protein) and the low-protein (LP) diet (7% of calories from protein), and were fed an isocaloric diet for the following 5 weeks. ***Results:*** Urinary protein and phosphate excretion were significantly greater in the HP diet group than in the LP diet group ($p < 0.05$). Serum parathyroid hormone and osteocalcin levels were significantly higher and lower, respectively, in the HP diet group ($p < 0.05$). Femur weight, femur mass index and femur calcium contents were significantly lower in the HP diet group than in the LP diet group ($p < 0.05$). Bone mineral density was significantly lower in the HP diet group than in the LP diet group ($p < 0.05$); however, bone mineral content did not differ between the two groups. ***Conclusion:*** We confirmed that an HP diet negatively affects bone mineral metabolism and bone density in ADR-induced nephrotic syndrome rats.

Introduction

Nephrotic syndrome is a renal dysfunctional state that can be caused by disorders of capillary vessels contained in the kidney nephrons [1]. Since Denis and Hobson [2] initially reported that a high level of calcium excretion and reduced calcium absorption with excessive urinary protein excretion are accompanied by hyperproteinuria in nephrotic syndrome and lead to hypocalcemia, many studies have been performed to investigate the mechanisms of impaired bone metabolism in nephrotic syndrome.

Generally, there are several factors that lead to hypocalcemia in nephrotic syndrome. Firstly, the secretion of 1-dehydroxylase in the kidney is decreased, which results in low levels of 1,25-$(OH)_2D_3$, which in turn promotes absorption of calcium from the intestine [3]. Secondly, proteinuria increases the loss of vitamin D-binding protein, which carries vitamin D and the precursor of 25(OH) D_3 [1, 4, 5]. Lastly, calcium reabsorption decreases in re-

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nal tubules as the glomerular filtration rate increases [6]. Hypocalcemia accelerates the secretion of parathyroid hormone (PTH) from the parathyroid glands, which causes hyperparathyroidism and increases the calcium resorption from bone to serum [5]. Hypocalcemia, induced by excessive urinary protein excretion and hyperparathyroidism, affects bone metabolism in nephrotic syndrome [5, 7]. In particular, it has significant effects on bone mineral density in growing children with nephrotic syndrome [8].

To date, many studies have reported that excessive dietary protein induces negative effects on bone metabolism, although these data are limited for nephrotic syndrome [9]. In fact, according to many studies, a high-protein (HP) diet builds up a large quantity of urea, increases urine volume, leads to renal hypertrophy and causes excretion of large amounts of minerals, including calcium, and vitamin D metabolites from the body [10, 11]. Excessive dietary animal protein in particular enhances calcium resorption from bones to neutralize sulfur-containing amino acids [12].

Although an HP diet accelerates proteinuria, which can cause a variety of symptoms of nephrotic syndrome, such as hypocalcemia, hyperparathyroidism and abnormal bone formation, dietary guidelines for nephrotic syndrome patients have not been established. We designed the present study to demonstrate the importance of dietary protein proportion on bone metabolism in nephrotic syndrome.

The purpose of this study was to investigate the effects of an HP diet on bone metabolism in rats with nephrotic syndrome induced by the injection of adriamycin (ADR), which degenerates renal function [13].

Materials and Methods

Animals and Diets

The experimental protocol was approved by the Animal Care and Use Review Committee of Kyung Hee University. A total of 24 4-week-old male Sprague-Dawley rats (150–170 g) were purchased from SLC Inc. (Shizuoka, Japan). Rats were housed in polycarbonate cages in temperature-controlled rooms (22 ± 2°C), with a relative humidity of 55 ± 5% and a 12-hour light/dark cycle. The rats were fed a pellet chow diet and were given water ad libitum for an adaptation period of 2 weeks.

After 2 weeks of adaptation in a metabolic cage, the animals (n = 24) received weekly 1.0-ml intraperitoneal injections of 2 mg/kg body weight ADR (doxorubicin, D1515, Sigma, St. Louis, Mo., USA) and were given water and AIN93G pellets (Research Diets, USA) ad libitum for 6 weeks to induce ADR-induced proteinuria, which has been well characterized as an experimental model for nephrotic syndrome. After nephrotic syndrome was confirmed from urinary and blood data (n = 8), ADR-treated animals (n = 16) were placed into one of two different groups, an HP diet group (n = 8; 30% of calories from protein and 7% from fat) or a low-protein (LP) diet group (n = 8; 7% of calories derived from protein and 40% from fat) for the remaining 5 weeks. Both diets contained equal numbers of calories to prevent effects due to differences in calorie intake.

Body Weight and Food Consumption

Body weight and food consumption were measured weekly. The food efficiency ratio was calculated using the following formula: [weight gain (g)/day]/[amount of food consumed (g)/day].

Urine Sample Analysis

For 24-hour urine collection at 0, 3 and 5 weeks, the animals were housed in metabolic cages. During the urinary collection period, rats were restrained from eating to avoid contamination of the urine; however, they were allowed free access to water. Instruments used for collecting urine were washed with 0.1 N HCl to prevent rot of the urine. Urine samples were centrifuged at 2,000 rpm for 15 min at room temperature, and the top layer was collected and stored at –70°C until analysis. The Bradford method was applied to determine urinary protein levels. Urinary calcium and inorganic phosphorus in the urine were determined by a chemistry autoanalyzer (Advia 1650, Bayer, Japan).

Blood Sample Analysis

Blood was collected at 0 and 5 weeks, following a 12-hour overnight fast. Rats were lightly anesthetized with ethyl ether, and blood samples were taken by heart puncture. Blood samples were immediately collected into EDTA-containing tubes and serum-separating tubes to separate the plasma and serum, respectively. Blood was centrifuged at 3,000 rpm for 15 min at 4°C, and the top layer (serum) was stored at –70°C until use in assays.

Serum ionized calcium was measured using an EML 100 (Radiometer, Copenhagen, Denmark), and serum calcium and inorganic phosphorus were determined by a chemistry autoanalyzer (Advia 1650, Bayer). Serum PTH, osteocalcin and osteopontin were measured in duplicate using Millipore's MILLIPLEX Bone Hormone Panel (Millipore, Billerica, Mass., USA). The plate was run on a Luminex 200 instrument using Bio-Plex Manager 4.1 standard software (Bio-Rad Laboratories, Hercules, Calif., USA). Alkaline phosphatase (ALP) was determined using commercial kits (Alkaline Phosphate Reagents, Bayer, Tarrytown, N.Y., USA) with an Advia 1650 (Bayer). Serum 1,25-$(OH)_2D_3$ was determined using commercial kits (1,25-Dihydroxy Vitamin D ^{125}I RIA kit, DiaSorin, Stillwater, Minn., USA) with a gamma counter (Cobra 5010 Series Quantum, Packard, Meriden, Conn., USA).

Analysis of Femur Samples

Bone samples were obtained after rats were sacrificed at 0 and 5 weeks. The right femur was collected and freed from soft tissue. The femurs were stored in 4% formalin. The ash content in the femur was measured by heating at 500°C for 2 h in an electric furnace, and then 10 ml of 6 N HCl solution was added and diluted to the adequate concentration for analysis of calcium. Calcium content in the femur was determined by atomic absorption spectrophotometry at 422.7 nm. Bone mineral density and bone mineral content were measured by dual-energy X-ray absorptiometry measurements (PlXlmus 2, GE Lunar Co., Madison, Mich., USA).

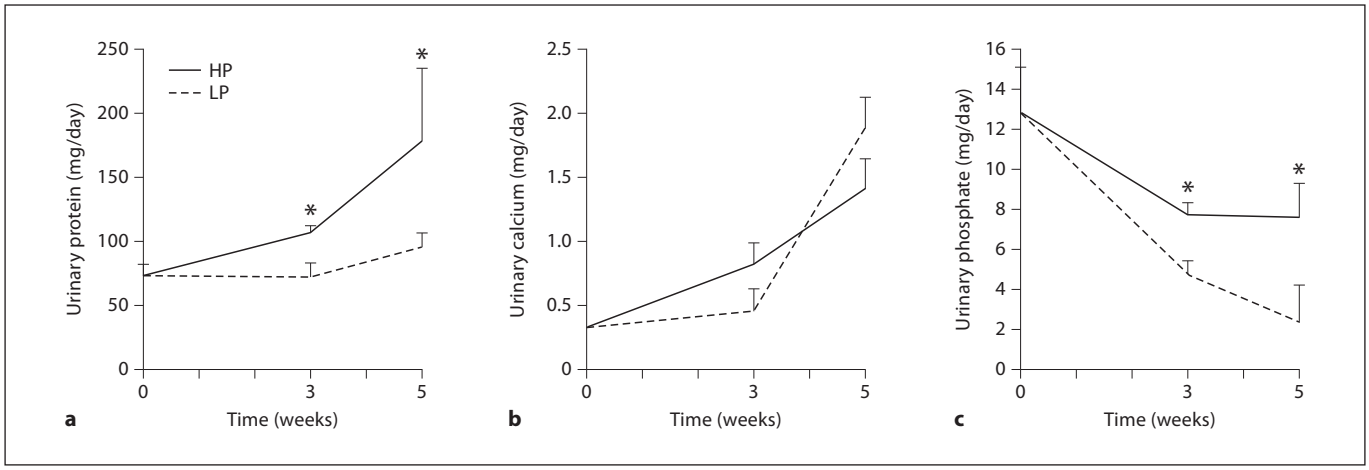

Fig. 1. Urinary excretion of protein (**a**), Ca (**b**) and P (**c**) in the HP and LP groups. * $p < 0.05$: statistical difference between the two groups (Student's t test).

Statistical Analysis

All measurements were performed in duplicate, and statistical calculations were performed with SPSS statistical software for Windows, version 13.0. All data are presented as means ± SD. Differences in measured parameters between the experimental groups were analyzed by Student's t test. The differences were considered to be significant when the p value was less than 0.05.

Results

Body Weight, Kidney Weight, Intake of Calories, Ca and P, and Food Efficiency Ratio

The mean values of body weight, kidney weight, intake of calories, Ca and P, Ca/P ratio and the food efficiency ratio are shown in table 1. Body weights of the rats in the HP and LP diet groups were not significantly different, neither at the beginning nor at the end of the experiment. Kidney weight was greater in the HP group (3.39 ± 0.58 g) than in the LP group (2.42 ± 0.15 g) at the end of the experiment. The daily calorie intakes and food efficiency ratios of the two groups did not differ due to the isocaloric intake. Intakes of Ca and P were higher in the HP group (72.1 ± 1.8 and 57.1 ± 1.4 mg/day, respectively) than in the LP group (61.3 ± 1.3 and 26.3 ± 0.54 mg/day, respectively); consequently, the Ca/P ratio was lower in the HP group (1.26:1) than in the LP group (2.33:1).

Table 1. Body weight, calorie intake, mineral intake, food efficiency ratio and kidney weight of the experimental groups

	HP	LP
Initial body weight, g	366.4 ± 16.4	374.3 ± 11.4
Final body weight, g	411.5 ± 12.1	415.5 ± 13.0
Weight gain, g	40.0 ± 8.5	41.2 ± 10.3
Kidney weight, g	0.85 ± 0.12	0.66 ± 0.04*
Calorie intake, kcal/day	58.0 ± 0.3	58.4 ± 0.5
Calcium intake, mg/day	72.1 ± 1.8	61.3 ± 1.3*
Phosphate intake, mg/day	57.1 ± 1.4	26.3 ± 0.5*
Calcium to phosphate ratio	1.26:1	2.33:1*
Food efficiency ratio	0.51 ± 0.12	0.43 ± 0.12

Kidney weight was measured as the total weight of the right kidney plus the left kidney. The food efficiency ratio was calculated as follows: [weight gain (g)]/[food consumed (g)/day]. * $p < 0.05$: statistical difference between the experimental groups (Student's t test).

Urinary Protein, Calcium and Phosphate Excretion

Figure 1 shows the urinary excretion of protein, Ca and P in the HP and LP groups. At 5 weeks, the urinary protein excretion had increased in both groups (by 245.4 and 131.8% in the HP and LP groups, respectively) compared with the initial levels ($p < 0.05$). There was a significant difference between the two groups at 3 and 5 weeks, with urinary excretion of protein in the HP group being significantly higher than that of the LP group (3 weeks: HP, 107.0 ± 5.8 mg/day, and LP, 71.6 ± 13.1 mg/day; 5 weeks: HP, 178.6 ± 55.9 mg/day, and LP, 95.9 ± 8.6 mg/day; $p < 0.05$).

The urinary Ca excretion had increased significantly in both groups when it was compared with the initial val-

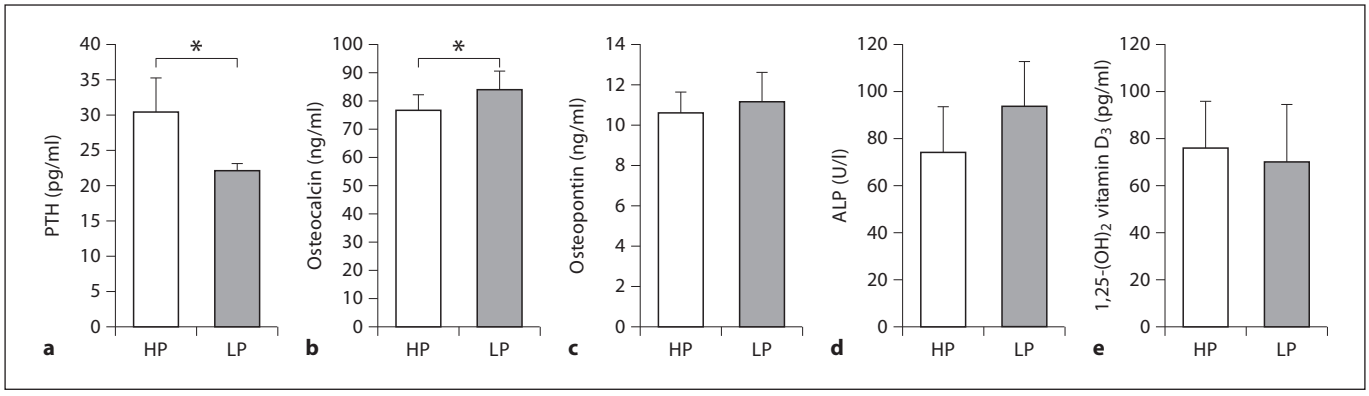

Fig. 2. Serum levels of PTH (**a**), osteocalcin (**b**), osteopontin (**c**), ALP (**d**) and 1,25-$(OH)_2$ vitamin D_3 (**e**). * $p < 0.05$ (Student's t test).

Table 2. Serum levels of calcium and phosphate in the experimental groups

	HP	LP
Serum Ca, mg/dl	9.4 ± 1.0	8.6 ± 0.5
Serum P, mg/dl	5.3 ± 0.8	6.0 ± 1.2
Ionized Ca, mM	1.20 ± 0.06	1.12 ± 0.06

Table 3. Femur wet weight, femur mass index and femur calcium content in the experimental groups

	HP	LP
Femur wet weight, g	0.703 ± 0.026	0.777 ± 0.028*
Femur mass index g/g body weight	0.171 ± 0.006	0.185 ± 0.005*
Femur Ca content, mg	75.1 ± 2.3	80.3 ± 5.1*

* $p < 0.05$: statistical difference between the experimental groups (Student's t test).

ues ($p < 0.05$). However, there was no significant difference between the two groups at 3 and 5 weeks (3 weeks: HP, 0.82 ± 0.16 mg/day, and LP, 0.45 ± 0.17 mg/day; 5 weeks: HP, 1.41 ± 0.23 mg/day, and LP, 1.89 ± 0.23 mg/day). In contrast, the excretion of urinary P in both groups decreased significantly throughout the experimental period ($p < 0.05$). There were significant differences in P excretion between the two groups at 3 and 5 weeks (3 weeks: HP, 7.73 ± 1.26 mg/day, and LP, 4.77 ± 1.15 mg/day; 5 weeks: HP, 7.62 ± 0.33 mg/day, and LP, 2.36 ± 3.19 mg/day; $p < 0.05$).

Serum Levels of Calcium and Phosphate

Serum levels of Ca, P and ionized Ca are shown in table 2. Serum Ca levels did not differ between the two groups (HP: 9.4 ± 1.0 mg/dl; LP: 8.6 ± 0.5 mg/dl). Similarly, there was no significant difference in the serum P levels between the groups (HP: 5.3 ± 0.8 mg/dl; LP: 6.0 ± 1.2 mg/dl). Additionally, the ionized Ca level in the HP group (1.20 ± 0.06 mM) did not differ from that in the LP group (1.12 ± 0.06 mM).

Serum Levels of PTH, Osteocalcin, Osteopontin, ALP and 1,25-$(OH)_2$ Vitamin D_3

Serum levels of PTH, osteocalcin, osteopontin, ALP and 1,25-$(OH)_2$ vitamin D_3 are shown in figure 2. The mean serum level of PTH in the HP group (30.3 ± 5.9 pg/ml) was significantly higher than that in the LP group (22.1 ± 1.1 pg/ml; $p < 0.05$). In contrast, the osteocalcin levels were significantly lower in the HP group (76.8 ± 5.2 ng/ml) than those in the LP group (83.6 ± 6.8 ng/ml; $p < 0.05$). No significant differences were shown in the serum levels of osteopontin, ALP and 1,25-$(OH)_2$ vitamin D_3 between the two groups.

Femur Wet Weight, Femur Mass Index and Calcium Content

Femur wet weight, femur mass index and Ca contents are shown in table 3. The weight of the femurs and femur mass index were significantly lower in the HP group (0.703 ± 0.026 g and 0.171 ± 0.006 g/g body weight, respectively) than those in the LP group (0.777 ± 0.028 g and 0.185 ± 0.005 g/g body weight, respectively; $p < 0.05$). In addition, femur Ca content was also significantly lower in the HP group (75.1 ± 2.3 g) than that in the LP group (80.3 ± 5.1 g; $p < 0.05$).

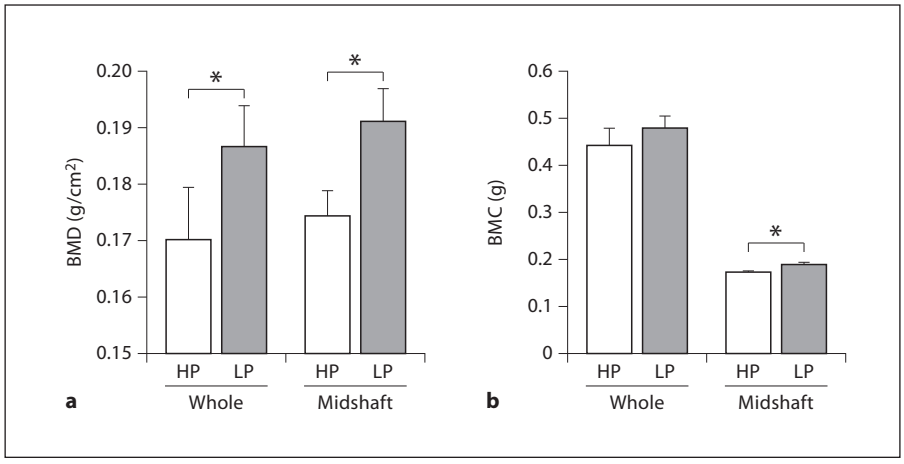

Fig. 3. Bone mineral density (BMD; **a**) and bone mineral content (BMC; **b**) in the whole femur and at midshaft (middle of the femur; measured area 3 cm^2). * $p < 0.05$ (Student's t test).

Femur Mineral Density and Femur Mineral Content in the Whole Femur and at Midshaft

The bone mineral density and bone mineral content in the whole femur and at midshaft are shown in figure 3. Bone mineral density in the whole femur and at midshaft was significantly lower in the HP group (0.1724 ± 0.0080 and 0.1743 ± 0.0045 g/cm^2, respectively) than that in the LP group (0.1864 ± 0.0073 and 0.1909 ± 0.0058 g/cm^2, respectively; $p < 0.05$). Bone mineral content in the whole femur in both groups did not differ (0.452 ± 0.033 and 0.481 ± 0.025 g, respectively); however, bone mineral content at the midshaft of the femur was significantly lower in the HP group (0.0318 ± 0.0018 g) than in the LP group (0.0360 ± 0.0017 g; $p < 0.05$).

Discussion

This study investigated the effects of an HP diet on bone formation in ADR-induced nephrotic syndrome rats. Nephrotic syndrome has many kinds of initial symptoms such as proteinuria, hypoalbuminemia and hypercholesterolemia [14]. If those symptoms last a long period of time, some cases can progress to chronic renal failure [15], which is generally known to develop the complications of bone disorders [16]. Previous studies have reported that abnormal bone remodeling and calcium metabolism occur in nephrotic syndrome patients [4, 5, 7], and these might be accelerated by an HP diet [9, 11, 12].

To demonstrate the importance of dietary protein for bone metabolism in nephrotic syndrome, the nephrotic syndrome was established in rats by the intraperitoneal administration of ADR, which inhibits DNA synthesis in not only cancer cells but also normal liver and kidney cells [13, 17]. After weekly injection of ADR for 6 weeks, we confirmed that nephrotic syndrome had developed by histology of the glomerulus (data has not shown). Thereafter, an HP (30% of the total calories) or LP (7% of the total calories) diet was given to the rats.

In this study, although the body weights of both groups were not different, the kidney weights in rats fed the HP diet were significantly greater than those in rats fed the LP diet. It is generally known that kidney hypertrophy is the early step in the process of nephron damage [18].

Proteinuria is a major index for the diagnosis of renal disease. In accordance with previous studies, this study also showed that urinary protein excretion increased as time passed in both groups, and its levels in the HP diet group were significantly higher than those in the LP diet group, showing the negative effects of an HP diet on renal function. As previously demonstrated in many studies, an excess consumption of dietary protein can increase glomerular pressure and filtration, which can cause deterioration of renal function [10, 19].

An HP diet has been shown to be related to the abnormal metabolism of calcium and phosphate in kidney disease patients [20, 21]. Human bodies preferentially maintain the homeostasis of the serum calcium concentrations; however, if the balance is skewed, hormones act to exchange calcium between blood and bone and control urinary calcium excretion. Thus, if the serum calcium level is low, then this mechanism negatively affects bone formation and remodeling [22]. In this study, the serum and urine levels of these minerals and several hormones that are related to bone metabolism were studied.

The serum levels of calcium and phosphate as well as urinary calcium excretion were not affected by the dietary protein in this study; however, urinary phosphate

excretion increased significantly in the HP diet group. Since an HP diet accelerates kidney hyperfiltration, generally serum calcium decreases and urinary calcium increases in nephrotic syndrome patients [23]. However, some studies failed to show any changes in serum calcium or phosphate levels [24, 25]. The homeostasis of serum minerals is maintained by a variety of hormones, such as PTH, and in the early stage of nephrotic syndrome, hormones may work to maintain the mineral balance in the serum [26].

In the case of hypocalcemia, PTH transfers calcium and phosphate from the bone to the blood and enforces calcium reabsorption as well as phosphate excretion from the kidney to maintain normal serum concentrations [27]. In the present study, the serum PTH levels were increased by the HP diet, and several previous studies showed the same results [11, 22]. It can be speculated that to maintain the homeostasis of calcium, calcium is released from the bone, constantly accompanied by high levels of serum PTH resulting from the HP diet-induced hyperfiltration of calcium. Moreover, it can be explained that the high level of serum PTH with the HP diet made no difference in the urinary calcium excretion compared to the LP diet because the higher serum PTH caused more reabsorption of calcium from the kidney, although the urinary protein excretion was significantly higher with the HP diet. We considered that the higher serum PTH level with the HP diet led to increased urinary phosphate excretion.

In bone mineral balance, osteocalcin, a noncollagenous protein secreted solely by osteoblasts, tends to increase when bone is formed [28]. Our results showed that the serum osteocalcin level was lowered by the HP diet, showing that an HP diet negatively affected or slowed down bone formation as compared to an LP diet. A number of studies have reported similar results [29]; however, some studies showed opposite results [30]. We suggest that this discrepancy could be a result of differences in kidney clearance or filtration ability among subjects [31].

The other hormone that influences bone metabolism is 1,25-dihydroxycholecalciferol [1,25-$(OH)_2D_3$]. It enhances the absorption of calcium and phosphate from the gastrointestinal tract. Its serum level decreases with severe proteinuria and progressive kidney damage. When the serum calcium level is low, PTH enhances the conversion of 25(OH)D to 1,25-$(OH)_2D_3$ in the kidney [32]. Decreased vitamin D levels induce secondary hyperparathyroidism and increase turnover of bone [5]. Several studies have reported that plasma vitamin D metabolite levels, including 1,25-$(OH)_2D_3$, decrease in nephrotic syndrome due to the excess loss of protein through urine [11, 22, 24]. However, a few studies did not show significant differences in those levels [33]. Our results showed that serum 1,25-$(OH)_2D_3$ levels were not affected by dietary protein, even though urinary protein excretion increased exponentially with the HP diet.

ALP is an enzyme found in all tissues, and in particularly high concentrations in the bone, and is secreted more when osteoblasts accumulate in the bone matrix [34]. In the present study, the serum ALP levels were not affected by the HP diet. In a previous study, the serum levels of ALP were lower in nephrotic syndrome rats fed an HP diet [11]. In contrast, a number of other previous studies have not shown any significant differences [35].

The effects of the HP diet on bone metabolism were evaluated by measuring femur weight, calcium content, mineral density and mineral content. Almost all of these measures were significantly lower in the rats on the HP diet than in those on the LP diet. This was likely due to the increased serum levels of PTH and urinary protein excretion with the HP diet. These findings indicate that an HP diet has deteriorative effects on bone metabolism. Other researchers have also reported that an HP diet caused bone loss resulting from excessive amounts of calcium and vitamin D metabolites in urine [9, 11, 36].

In this study, the amount of dietary calcium and phosphate and the ratio of calcium to phosphate were higher and lower, respectively, with the HP diet than with the LP diet. This could lead to increased calcium excretion with the HP diet [37]. In particular, excessive intake of phosphate from an HP diet accelerates kidney dysfunctions, such as dysregulation of the calcium and phosphate balance, and limits the conversion of 25(OH)D into 1,25-$(OH)_2D_3$ [38, 39]. In this experiment, the histology of the glomerulus in rats on the HP diet showed that the boundary of the glomerulus was weaker and renal tubules had bigger holes than in rats on the LP diet (data not shown). Therefore, an HP diet in nephrotic syndrome rats influenced kidney histology, resulting in abnormal bone metabolism.

In conclusion, an HP diet induced an increase in urinary protein and phosphate as well as serum PTH levels in nephrotic syndrome rats. These changes may cause the changes in the serum calcium concentration and vitamin D metabolites and, finally, decreases in femur weight, calcium content and bone mineral density of the femur. Thus, we could conclude that an HP diet might negatively affect kidney function and bone formation in ADR-induced nephrotic syndrome rats.

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