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Abstract:

In this study, we aimed to investigate the physiological and skeletal responses of broiler chicks to graded dietary reductions in dietary non-phytate phosphorus (NPP) at constant calcium (Ca) levels. The chicks were fed a control diet until day 7 and then fed (days 8–21) either a control diet (NPP 0.48%) or NPP-deficient diets (0.37, 0.26, or 0.15%) with a constant Ca content (0.96%). Serum Ca, phosphorus (P), and 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2\text{D}_3$] levels were analyzed; intestinal and renal P- and Ca-related transporter gene expression was quantified; and tibial ash content, strength, and growth plate (GP) histology were evaluated. The results revealed that graded reductions in dietary NPP at a constant Ca level were associated with reduced serum P levels and increased serum Ca and $1,25(\text{OH})_2\text{D}_3$ levels. The mRNA expression of *CALB1*, *OCN*, *CLDN12*, *SLC34A2*, and *SLC34A1* was upregulated; however, despite the upregulation of these mineral transport-related genes, serum P concentrations remained low under severe dietary NPP restriction. The tibial size, strength, and ash content decreased, and thickening of the GP, indicative of hypophosphatemic rickets, was observed. The serum Ca:P ratio increased with the dietary Ca:NPP ratio and appeared to plateau under severe NPP restriction, suggesting a threshold-like response within the range of dietary conditions tested. In conclusion, graded reductions in dietary NPP were associated with increased circulating $1,25(\text{OH})_2\text{D}_3$ concentrations and upregulation of Ca- and P-transport-related genes, while systemic P availability remained low under severe NPP restriction. The serum Ca:P ratio may have potential as an exploratory marker of dietary mineral imbalance in broilers.

Keywords: Non-phytate phosphorus, Mineral homeostasis, Serum Ca:P ratio, Tibial mineralization, Rickets, Mineral transporter gene expression

Introduction

The dynamic relationship between phosphorus (P) and calcium (Ca) plays a pivotal role in poultry nutrition, particularly in supporting skeletal growth and development. Inadequate levels of these minerals during the early growth stages impair the skeletal and physiological growth of broiler chickens [1,2]. Bone mineralization disorders, such as rickets, remain an economically significant concern in the poultry industry. These disorders are important in rapidly growing broiler chickens, in which their high incidence and rapid progression are strongly associated with intensive growth rates and skeletal development [3–5]. The majority of P in plant-derived feed ingredients exists in the form of phytate, which is largely indigestible by monogastric animals, including broilers, because of insufficient endogenous phytase activity [6]. In poultry nutrition, non-phytate phosphorus (NPP) is defined as the fraction of

47 dietary P that is not bound to phytate and is readily available for absorption. Therefore, dietary P is expressed as NPP.
48 Dietary P requirements in broilers are commonly expressed and formulated based on NPP, rather than total P, to
49 accurately reflect the bioavailable P supply in the diet. Despite extensive research on mineral-related skeletal disorders,
50 the endocrine mechanisms linking dietary P deficiency to skeletal abnormalities remain poorly understood.

51 P plays a crucial role in bone formation, energy metabolism, and other cellular processes. It is primarily
52 absorbed in the small intestine, and its homeostasis is maintained through coordinated intestinal absorption and renal
53 excretion. Moreover, it closely interacts with Ca metabolism. P and Ca absorption in the intestine occurs via both
54 passive and active pathways [7,8]. Passive transport relies on paracellular movement along concentration gradients
55 [8,9] and is regulated by tight junction proteins, such as occludin (*OCLN*) and claudin-12 (*CLDN12*), whose expression
56 is sensitive to dietary mineral status [10–12]. Active transport involves the functions of sodium-phosphate
57 cotransporter type IIb (*SLC34A2*) for P and calbindin D-28k (*CALB1*) for Ca, and their expression increases in
58 response to dietary deficiencies of the respective minerals [13–15]. In the kidneys, P reabsorption is primarily mediated
59 by sodium-phosphate cotransporter type IIa (*SLC34A1*) in the proximal tubules, contributing significantly to systemic
60 P homeostasis [2,7,16]. Concurrently, renal Ca reabsorption is facilitated by *CALB1*, which plays a key role in
61 intracellular Ca transport [2].

62 In general, hypertrophic chondrocytes (mature cartilage cells) in the growth plate (GP) undergo
63 mineralization and are subsequently replaced by bone-forming cells (osteoblasts), enabling endochondral ossification.
64 However, the dietary deficiencies of P, Ca, or vitamin D impair this process, resulting in skeletal abnormalities, such
65 as rickets [17,18]. Hypophosphatemic (P-deficiency) rickets is characterized by a marked thickening of the tibial GP
66 and disrupted endochondral ossification [4]. Bone mineralization relies on the deposition of P and Ca into the organic
67 bone matrix, forming hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) with an approximate Ca:P mass ratio of 2.0 [19], which
68 provides structural rigidity to the bones [20,21]. An imbalance in mineral supply leads to defective mineral deposition,
69 abnormal chondrocyte organization, and reduced bone strength [22,23]. Moreover, dietary P or Ca deficiency
70 compromises bone integrity and alters the concentrations of these minerals in the bloodstream, indicating systemic
71 disturbances in mineral homeostasis and skeletal development [2,10,12].

72 Although previous studies have investigated the effects of dietary P deficiency on mineral metabolism,
73 mineral transporter expression, and bone development in broilers [2,4,23], the concurrent physiological,
74 molecular, and skeletal responses to graded reductions in dietary NPP have not been fully characterized. In
75 particular, the relationships among circulating mineral concentrations, 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2\text{D}_3$]

levels, intestinal and renal mineral transport-related gene expression, and skeletal development under progressively reduced NPP conditions remain unclear.

In this study, we aimed to investigate the physiological, molecular, and skeletal responses of broiler chicks to graded reductions in dietary NPP at a constant Ca level by evaluating serum mineral profiles, circulating 1,25(OH)₂D₃ levels, tibial structural characteristics, and expression of intestinal and renal mineral transport markers. We hypothesized that graded reductions in dietary NPP would be associated with coordinated changes in circulating mineral concentrations, 1,25(OH)₂D₃ levels, mineral transport-related gene expression, and tibial development.

Materials and Methods

In this study, the animal experimental protocol used for the care of chicks and all experimental procedures were approved by the Committee for Animal Experiments at Niigata University, Japan (reference numbers: SA00132 and SA01391).

Animals, diets, and experimental plan

Twenty-eight male Ross 308 broiler chicks were housed in individual cages throughout the experimental period. Each cage was equipped with an individual feeder and drinker, and feed and water were provided *ad libitum*. Room temperature was maintained according to the Ross 308 broiler management guidelines [24], and a 23-h lighting schedule was applied throughout the experimental period. From days 0 to 7, all chicks were fed the control diet. On day 8, the chicks were randomly assigned to four dietary treatments, with seven individually housed chicks per treatment, and maintained until day 21. Because each chick was housed separately, received its assigned diet independently, and was sampled individually, each bird was considered an independent experimental unit ($n = 7$ per treatment). Diets were formulated based on the Ross 308 Nutrition Specifications [24], with Ca maintained at 0.96% and NPP progressively reduced from 0.48% in the Control diet to 0.37, 0.26, and 0.15% in the Low P1, Low P2, and Super Low P diets, respectively. Consequently, the dietary Ca:NPP ratio increased as NPP decreased. All diets were isoenergetic and isonitrogenous (3.00 Mcal/kg metabolizable energy and 23.0% crude protein). The detailed dietary composition is provided in Supplementary Table 1.

Feed and water were provided *ad libitum* until blood collection. This condition was selected to avoid introducing fasting as an additional factor affecting circulating mineral concentrations, as fasting duration has been reported to affect serum Ca and P concentrations in broiler chickens [25]. Body weight (g) was individually measured

on day 21 of age using a digital scale before sample collection. The birds were humanely euthanized according to the approved ethical guidelines. The tibiae were excised and cleaned of residual connective tissues. The right tibiae were stored at -20°C until further bone analysis, whereas the proximal ends of the left tibiae were fixed in 10% neutral-buffered formalin for 2 days for histological analysis. The duodenum, jejunum, ileum, and kidneys were excised immediately, quick-frozen in liquid nitrogen, and stored at -80°C for quantitative real-time polymerase chain reaction quantitative PCR (qPCR) analyses. Blood samples were centrifuged at $1,470 \times g$ for 15 min to obtain serum, which was aliquoted and stored at -80°C until Ca, P, and $1,25(\text{OH})_2\text{D}_3$ concentrations were determined.

Measurement of serum Ca, P, and $1,25(\text{OH})_2\text{D}_3$ concentrations

The obtained serum was thawed, and Ca (mg/dL) and P (mg/dL) concentrations were measured. Ca E Test and Phosphor C Test kits (Wako Pure Chemical Industries Ltd., Tokyo, Japan) were used to determine Ca and P concentrations, respectively, according to the manufacturer's instructions. Ca and P concentrations were measured spectrophotometrically at 610 nm and 750 nm, respectively (Ultrospec 2000 UV/ Visible Spectrophotometer; Pharmacia Biotech, Tokyo, Japan). The serum concentrations of $1,25(\text{OH})_2\text{D}_3$ (pg/mL) were assayed using a $1,25(\text{OH})_2\text{D}$ Radioimmunoassay Kit FR (Fujirebio Inc., Tokyo, Japan) according to the manufacturer's instructions.

Bone strength

Before bone strength analysis, the length (mm) and midshaft width (mm) of each tibia were measured and recorded using a digital caliper. To measure bone strength, the right tibia was thawed at room temperature, following which bone strength was measured using the three-point bending method with a Universal Testing Instrument (Tensilon RTC-1225AS; A&D, Tokyo, Japan). The measurement conditions were as follows: the cell load was 2,500 N and was applied at a rate of 10 mm/min. The distance between the fulcrums was set at 3.0 cm.

Ash content in tibia bones

The tibia bones were dried overnight at 105°C . Empty crucibles were pre-ashed, cooled, and weighed before use. The dried bones were placed in the crucibles and ashed at 600°C for 6 h in a muffle furnace. After cooling, the crucibles were reweighed, and the ash content was calculated both in grams and as a percentage of dry bone weight.

Staining method

After formalin fixation, the tibiae were decalcified overnight in Kalkitox (Wako Pure Chemical Industries Ltd., Japan). After decalcification, the bones were dehydrated through an ascending series of ethanol solutions and embedded in paraffin for sectioning. The samples were sectioned at a thickness of 5 μ m using a microtome. The sectioned samples were stained with Alcian blue and Meyer's hematoxylin, dehydrated through an ascending alcohol series, permeabilized with Lemosol A (Wako Pure Chemical Industries Ltd.), and sealed in a soft mount (Wako Pure Chemical Industries Ltd.). The Alcian blue-stained histological sections were scanned, and the scale was calibrated against a 1-mm ruler. The thickness (mm) of the GP was measured using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). For each sample, the measurements were recorded at three distinct locations (left, center, and right regions of the GP), and the mean value of these measurements was used as the representative thickness of the GP.

Total RNA extraction, cDNA synthesis, and qPCR analyses

Total RNA from the intestines and kidneys was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan), and cDNA was synthesized from this total RNA using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer's instructions. qPCR was performed using the THUNDERBIRD Next SYBR qPCR Mix (Toyobo Co. Ltd., Japan). The PCR was performed for 40 cycles under the following cycling conditions: initial denaturation for 3 min at 95°C; denaturation for 10 s at 95°C; and annealing and extension for 30 s at 55°C. The relative mRNA expression levels of each gene were calculated using the $\Delta\Delta C_T$ method and normalized to the *ACTB* expression levels. *ACTB* was selected as the housekeeping gene because of its stable expression across all treatments. Values are expressed in arbitrary units (AU), relative to those of *ACTB*. The primer sequences and corresponding National Center for Biotechnology Information accession numbers are provided in Supplementary Table 2.

Statistical analyses

All values are presented as the mean $\pm$ SEM. Statistical analyses were performed using the GraphPad Prism 11 software (GraphPad Software Inc., Boston, MA, USA). Differences between different groups were analyzed using a one-way analysis of variance, followed by Tukey's multiple comparison test. Each bird was considered an independent experimental unit. Statistical significance was set at $p < 0.05$.

The relationship between the dietary Ca:NPP ratio and serum Ca:P ratio was evaluated using simple linear regression, and the coefficient of determination (R^2) and the corresponding p -values were calculated. In addition, a linear broken-line regression model was used to estimate the breakpoint (R) and its 95% confidence interval (CI).

Results

Growth performance

No mortality occurred during the experimental period. Body weight was measured on day 21 in all four groups. Compared with the Control group, body weight did not differ significantly in the Low P1 group ($p = 0.466$), whereas it was significantly lower in the Low P2 group ($p < 0.0001$) and the Super Low P group ($p < 0.0001$) (Table 1).

Serum Ca, P, and 1,25(OH) $_2$ D $_3$ concentrations

Serum Ca, P, and 1,25(OH) $_2$ D $_3$ levels differed significantly between the groups (Fig. 1). Serum Ca levels were significantly higher in the Low P2 ($p < 0.0001$) and Super Low P ($p < 0.0001$) groups than in the Control and Low P1 groups (Fig. 1A). Serum P levels were significantly lower in the Low P1 ($p = 0.0001$), Low P2 ($p < 0.0001$), and Super Low P ($p < 0.0001$) groups than in the Control group (Fig. 1B). Moreover, serum 1,25(OH) $_2$ D $_3$ levels were significantly higher in the Low P2 ($p = 0.0025$) and Super Low P ($p < 0.0001$) groups than in the Control group (Fig. 1C).

Bone characteristics

Tibial length, width, strength, ash content, and GP thickness were measured to assess the effects of varying NPP levels on the skeletal development of broiler chicks. Bone length was significantly lower in chicks fed the Low P2 ($p < 0.0001$) and Super Low P ($p < 0.0001$) diets than in those fed the Control diet. Bone width was also significantly lower in chicks fed the Low P2 ($p = 0.0044$) and Super Low P ($p < 0.0001$) diets than in those fed the Control diet (Table 1). Both bone strength and ash content in the tibia were significantly lower in the Low P1 ($p < 0.0001$), Low P2 ($p < 0.0001$), and Super Low P ($p < 0.0001$) groups than in the Control group (Table 1). The thickness of the tibial GP was significantly greater in the Low P2 ($p < 0.0001$) and Super Low P ($p < 0.0001$) groups than in the Control group (Table 1). In particular, a pronounced accumulation of hypertrophic chondrocytes was observed in chicks fed Low P2 and Super Low P diets, likely resulting in the increased GP thickness (Fig. 2).

Relative mRNA expression levels of *CALB1*, *SLC34A2*, *SLC34A1*, *OCN*, and *CLDN12* in the duodenum, jejunum, ileum, and kidney tissues

The mRNA expression levels of *CALB1* and *SLC34A2* in the duodenum were significantly higher in the Low P2 ($p < 0.0001$) and Super Low P ($p < 0.0001$) groups (Figs. 3A-I and 3A-II), and the mRNA expression of *OCN* was also higher in the Low P2 ($p = 0.0060$) and Super Low P ($p = 0.0034$) groups (Fig. 3A-III). *CLDN12* mRNA expression was higher in the Low P2 ($p = 0.0080$) and Super Low P ($p < 0.0001$) groups than in the Control group ($p < 0.05$) (Fig. 3A-IV).

In the jejunum, the mRNA expression level of *CALB1* was significantly higher in the Low P1 ($p = 0.0150$), Low P2 ($p = 0.0001$), and Super Low P ($p < 0.0001$) groups than in the Control group (Fig. 3B-I). Similarly, *SLC34A2* mRNA expression was significantly higher in the Low P1 ($p = 0.0001$), Low P2 ($p < 0.0001$), and Super Low P ($p < 0.0001$) groups than in the Control group (Fig. 3B-II). Jejunal *OCN* mRNA expression was higher in the Low P2 ($p = 0.0126$) and Super Low P ($p = 0.0005$) groups, and *CLDN12* expression was similarly higher in the Low P1 ($p = 0.0015$), Low P2 ($p = 0.0001$), and Super Low P ($p < 0.0001$) groups than in the Control group (Figs. 3B-III and 3B-IV).

The average *CALB1* mRNA expression levels in the ileum of the birds in the Low P2 ($p < 0.0001$) and Super Low P ($p = 0.0004$) groups were significantly elevated compared with those in the Control group (Fig. 3C-I). Similarly, *SLC34A2* mRNA expression in the ileum was significantly higher in the Low P2 ($p = 0.0307$) and Super Low P ($p < 0.0001$) groups than in the Control group (Fig. 3C-II). *OCN* mRNA expression was also significantly higher in the Low P2 ($p = 0.0009$) and Super Low P ($p = 0.0004$) groups than in the Control group (Fig. 3C-III). Likewise, *CLDN12* mRNA expression was significantly higher in the Low P2 ($p = 0.0265$) and Super Low P ($p = 0.0301$) groups than in the Control group (Fig. 3C-IV).

These results indicate that the mRNA expression of *CALB1*, *SLC34A2*, *OCN*, and *CLDN12* increases in response to relatively lower dietary NPP levels. The mRNA expression of Ca and P transport-related genes increased progressively in the following order: Control group < Low P1 group < Low P2 group $\leq$ Super Low P group.

Renal *CALB1* mRNA expression increased significantly with decreasing dietary NPP levels, with the Low P1 ($p = 0.0069$), Low P2 ($p < 0.0001$), and Super Low P ($p < 0.0001$) groups exhibiting higher *CALB1* expression than in the Control group (Fig. 4A). Renal *SLC34A1* mRNA expression exhibited an upregulation with decreasing

207 dietary NPP levels, with the Low P2 ($p = 0.0002$) and Super Low P ($p < 0.0001$) groups exhibiting significantly higher
208 *SLC34A1* expression levels than in the Control group (Fig. 4B).

210 **Relationship between dietary Ca:NPP and serum Ca:P ratios**

211 The relationship between the dietary Ca:NPP ratio and serum Ca:P ratio was first evaluated using a simple linear
212 regression model, which revealed a significant positive association ($p < 0.05$). However, the model exhibited only a
213 moderate fit to the data ($R^2 = 0.63$), suggesting that the relationship cannot be adequately described using a simple
214 linear model.

215 A linear broken-line regression model was used to further characterize the response patterns. Based on the
216 model results, we identified a breakpoint (R) at 3.44 (95% CI: 3.16–3.86), indicating that the serum Ca:P ratio increases
217 with the dietary Ca:NPP ratio up to this point and then plateaus. The broken-line model provided a better fit to the
218 data ($R^2 = 0.95$) than the simple linear regression model, suggesting a threshold-like response within the range
219 of dietary Ca:NPP ratios tested (Fig. 5).

220 **Discussion**

221 In this study, we examined the responses of serum mineral markers, intestinal and renal mineral transport-related gene
222 expression, and tibial characteristics to graded reductions in dietary NPP at a constant Ca level in broiler chickens. As
223 reported previously [23], serum P concentrations decreased in response to reduced dietary NPP content. In contrast,
224 serum Ca concentrations increased concomitantly with a decrease in dietary NPP levels, despite a constant dietary Ca
225 concentration, which is consistent with previous reports [23,26]. This physiological response is likely attributable to
226 hypophosphatemia, which may stimulate renal 1α -hydroxylase activity and promote the production of the active form
227 of vitamin D, $1,25(\text{OH})_2\text{D}_3$, which is known to regulate intestinal and renal Ca transport [27–29]. Consistent with this
228 mechanism, the results obtained in our study demonstrated a significant elevation in circulating $1,25(\text{OH})_2\text{D}_3$ levels,
229 accompanied by an increased mRNA expression of *CALB1*, a key mediator of active Ca transport in the intestine [30–
230 32], and of the tight junction–related genes *OCN* and *CLDN12*, which are involved in passive Ca transport [32]. In
231 addition, increased *CALB1* mRNA expression was observed in the kidneys of chicks fed the NPP-restricted diets.
232 Under normal physiological conditions, hypercalcemia is associated with a reduction in circulating $1,25(\text{OH})_2\text{D}_3$
233 concentrations, accompanied by the suppression of intestinal Ca absorption and renal Ca reabsorption, thereby
234 ensuring a tight regulation of serum Ca levels [33]. However, despite the generally tight regulation of serum Ca

concentrations [34], the present study showed increased serum Ca concentrations under severe dietary NPP restriction, accompanied by persistently low serum P concentrations.

In the present study, serum P concentrations did not recover despite a significant upregulation of *SLC34A2* and *SLC34A1* mRNA expression in the intestine and kidney, respectively, consistent with a previous report on intestinal P transport [35]. These findings indicate that the upregulation of P transport-related genes was insufficient to maintain circulating P concentrations under severe NPP restriction. In general, hypophosphatemia promotes the production of 1,25(OH)₂D₃ by suppressing the secretion of the bone-derived hormone fibroblast growth factor 23 (FGF23), accompanied by enhanced renal 1 α -hydroxylase activity [36]. The increase in circulating 1,25(OH)₂D₃ concentration observed in the present study may reflect an endocrine-compensatory response to hypophosphatemia. However, because FGF23 and PTH were not directly measured in the present study, their involvement in the observed responses cannot be established. Therefore, the endocrine pathways discussed above should be regarded as potential mechanisms inferred from the observed changes in circulating 1,25(OH)₂D₃ and previous studies [28,37]. Future studies involving direct measurements of FGF23, PTH, and related endocrine regulators are required to clarify these mechanisms.

The histological examination of the proximal end of the tibia in this study revealed a marked accumulation of hypertrophic chondrocytes. This finding is characteristic of hypophosphatemic rickets and is likely attributable to impaired hydroxyapatite deposition within the cartilage matrix surrounding hypertrophic chondrocytes under reduced dietary NPP conditions [3–5]. Consequently, the subsequent replacement of the cartilage with trabecular bone through coordinated osteoclastic and osteoblastic activities appears to be disrupted. Consistent with these histological abnormalities, bone length, width, and ash content were significantly reduced in the Low P2 and Super Low P groups. Collectively, these findings indicate that hydroxyapatite deposition in the bone is severely impaired, preventing normal bone growth and mineral accrual [39]. These skeletal abnormalities were accompanied by a marked reduction in final body weight, particularly in the Super Low P group. However, because feed intake and FCR were not recorded, the present study cannot determine whether the lower body weight was attributable to reduced voluntary feed intake, altered nutrient utilization, skeletal impairment, or a combination of these factors. In addition, differences in dietary ingredient composition among treatments, including changes in fish meal, corn gluten meal, animal fat, and Ca sources, may have affected amino acid profiles, trace mineral supply, energy contribution, or palatability and therefore represent additional potential confounding factors.

Furthermore, in this study, a linear broken-line regression model was used to elucidate the relationship between the dietary Ca:NPP ratio and serum Ca:P ratio. When the dietary Ca:NPP ratio was approximately 2.0, the serum Ca:P ratio remained within the previously reported physiological range (1.5–2.0), suggesting effective homeostatic regulation [2]. In contrast, as the dietary Ca:NPP ratio increased, the serum Ca:P ratio also increased markedly and exceeded the normal range. Specifically, serum Ca:P ratios of 6.26 and 6.34 were observed in the Low P2 and Super Low P groups, respectively, with no further substantial increase observed at higher dietary Ca:NPP ratios. Notably, beyond a dietary Ca:NPP ratio of approximately 3.44, the serum Ca:P ratio plateaued, suggesting that further increases in the dietary Ca:NPP ratio did not substantially increase the serum Ca:P ratio within the tested range. These findings suggest that the relationship between dietary mineral balance and circulating Ca and P concentrations may change as dietary NPP decreases and the Ca:NPP ratio increases. However, because dietary Ca was maintained at a constant level while NPP was progressively reduced, the dietary Ca:NPP ratio increased concurrently with decreasing NPP concentrations. Therefore, the effects of reduced NPP concentration cannot be separated from those of the increased Ca:NPP ratio in the present experimental design, and the observed responses should not be attributed solely to P deficiency. The broken-line model provided a good fit to the data ($R^2 = 0.95$), suggesting a threshold-like response within the range of dietary Ca:NPP ratios tested in this study. However, because the breakpoint was estimated from only four dietary Ca:NPP ratios, it should be regarded as exploratory rather than as a definitive physiological threshold.

Consistent with this interpretation, circulating $1,25(\text{OH})_2\text{D}_3$ concentrations also plateaued at the Low P2 levels, with no significant difference observed between the Low P2 and Super Low P groups in the present study. In human clinical practice, the serum Ca:P ratio is used as a simple screening indicator of primary hyperparathyroidism and is regarded as an integrated marker of the disruption of Ca and P homeostasis [40]. The use of phytase in the poultry industry has become widespread as a strategy to reduce environmental P loading in poultry feces. However, its efficacy depends on multiple factors, including the dietary Ca:NPP ratio, phytate P content, digestibility of Ca and P, and chicken breed and health status [41,42]. Therefore, the serum Ca:P ratio may have potential as an exploratory screening marker of dietary mineral imbalance in broilers and may provide useful information for evaluating dietary Ca and P balance and P-utilization strategies, including phytase supplementation. Further studies are needed to validate its practical applicability under a wider range of dietary and production conditions.

Taken together, graded reductions in dietary NPP were associated with hypophosphatemia, increased circulating $1,25(\text{OH})_2\text{D}_3$ concentrations, and upregulation of intestinal and renal mineral transport-related genes. These changes were accompanied by impaired tibial mineralization and altered growth plate morphology. Although

these findings are consistent with an endocrine-mediated adaptive response to reduced dietary NPP, the underlying endocrine mechanisms remain to be established because key regulators, including FGF23 and PTH, were not directly measured. Furthermore, the serum Ca:P ratio may reflect the disruption of Ca and P homeostasis under reduced dietary NPP conditions, although further evaluation is required to determine its usefulness in assessing dietary Ca and NPP formulations.

Acknowledgments

Not applicable.

Supplementary Material

Supplementary Table 1. Ingredient and nutrient composition (%) of diets containing different concentrations of non-phytate phosphorus (NPP). Control (NPP 0.48%), Low P1 (NPP 0.37%), Low P2 (NPP 0.26%), Super Low P (NPP 0.15%).

Supplementary Table 2. Sequences of primers used for quantitative PCR.

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Tables

Table 1. Effect of graded levels of non-phytate phosphorus (NPP) on growth performance and bone characteristics of broiler chicks

	Con	LP1	LP2	SLP	SEM	<i>p</i> -value
Body weight (g)	993.7 ^a	871.6 ^a	690.1 ^b	424.7 ^c	31.0	<0.0001
Tibia length (mm)	79.3 ^a	78.9 ^a	72.7 ^b	67.7 ^c	3.4	<0.0001
Tibia width (mm)	6.9 ^a	6.5 ^{ab}	5.9 ^{bc}	5.2 ^c	0.5	<0.0001
Tibia ash content (%)	36.9 ^a	27.2 ^b	22.9 ^c	18.3 ^d	6.3	<0.0001
Bone strength (N)	261.1 ^a	170.6 ^b	73.8 ^c	30.0 ^d	43.7	<0.0001
Growth plate thickness (mm)	6.3 ^b	6.5 ^b	9.7 ^a	8.4 ^a	0.6	<0.0001

Data were analyzed using one-way analysis of variance to evaluate the effect of dietary NPP levels. When a significant effect was detected, treatment means were separated using Tukey's multiple comparison test. Means lacking a common superscript letter differ significantly according to Tukey's multiple comparison test ($p < 0.05$). The results are presented as means, and the SEM is shown in a separate column.

Con, Control; LP1, Low P1; LP2, Low P2; SLP, Super Low P.

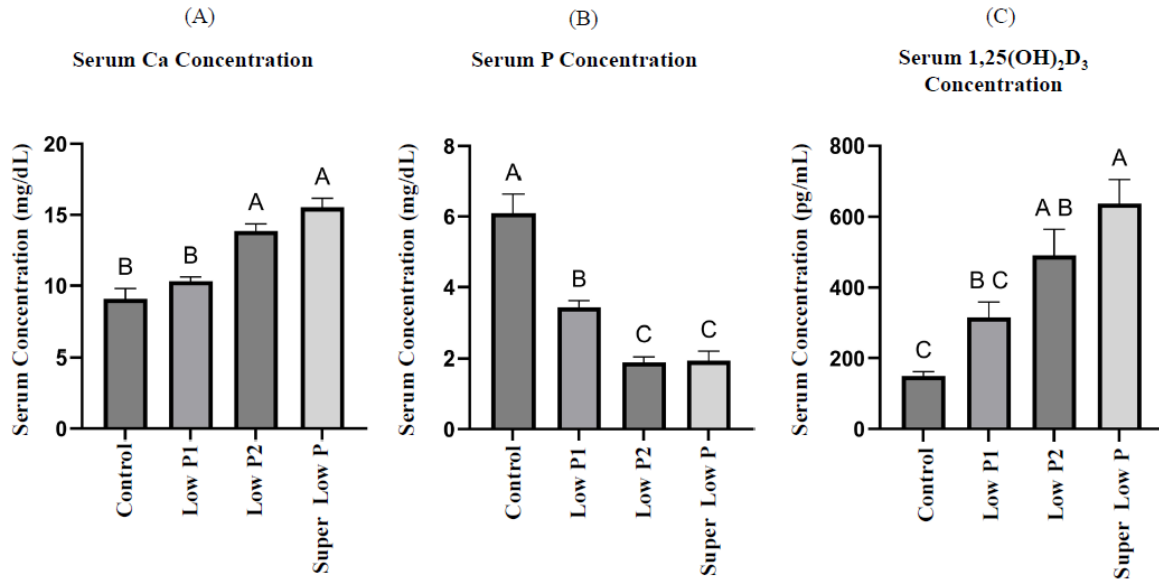


Fig. 1. Serum concentrations of calcium(A), phosphorus (B), and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (C). Bars lacking a common superscript letter differ significantly ($p < 0.05$).

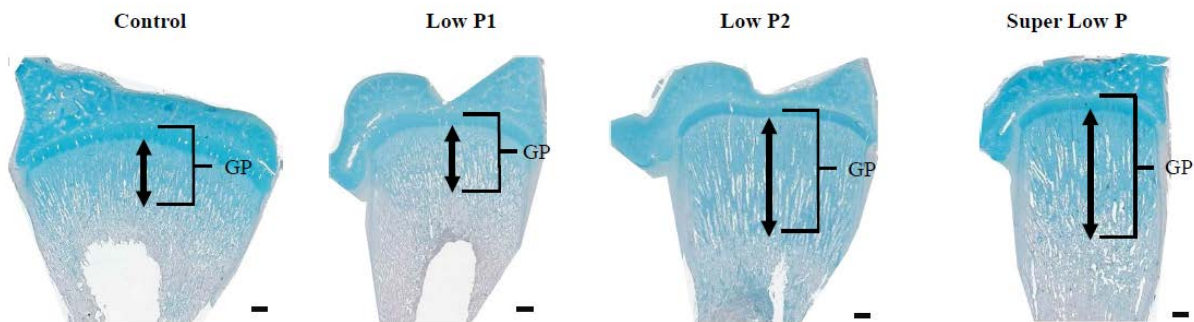
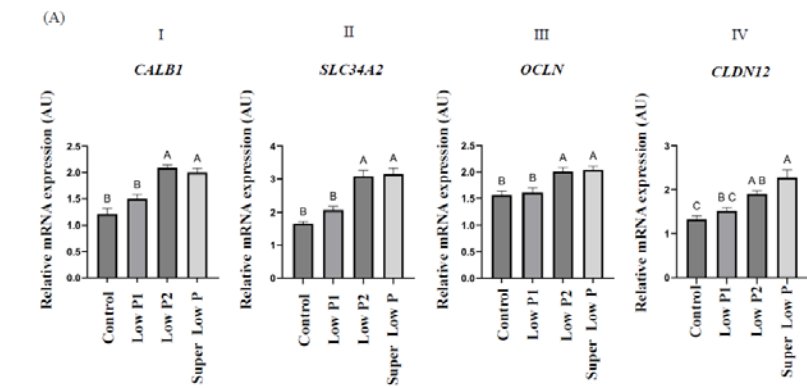
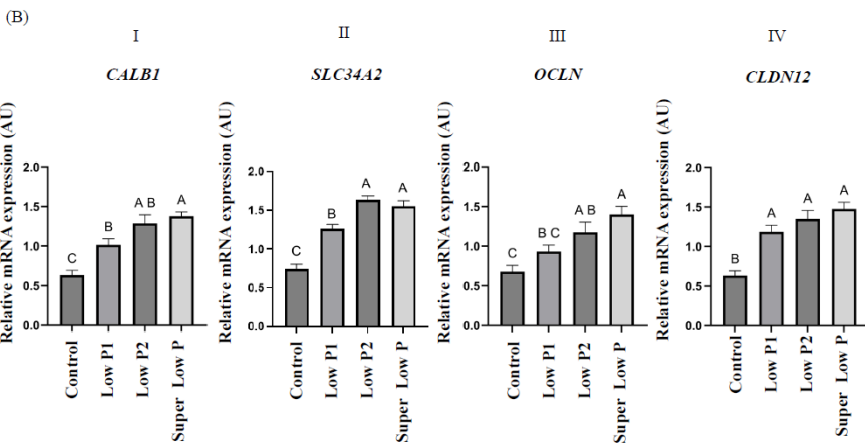


Fig 2. Histological changes in the proximal tibial growth plate (GP) under dietary non-phytate phosphorus restriction. Histological analysis shows elongation of the GP in the Low P2 and Super Low P diet groups. In particular, a pronounced accumulation of hypertrophic chondrocytes (double-headed arrows) is observed in these groups. Alcian blue staining. Bar = 1mm.

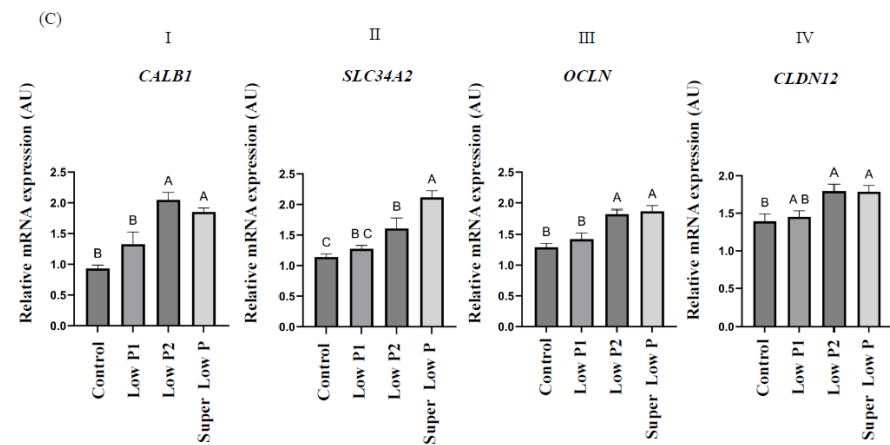
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Fig 3.(A-C) mRNA expression levels of Calbindin-D28k (*CALB1*), NaPi-IIb (*SLC34A2*), Occludin(*OCLN*), and Claudin-12 (*CLDN12*) in the duodenum (A), jejunum (B), and ileum (C). Subpanels (I-IV) correspond to *CALB1*, *SLC34A2*, *OCLN*, and *CLDN12*, respectively. Values are

means $\pm$ SEM (n = 7). Bars lacking a common superscript letter differ significantly ($p < 0.05$). The levels are represented as arbitrary units (AU).

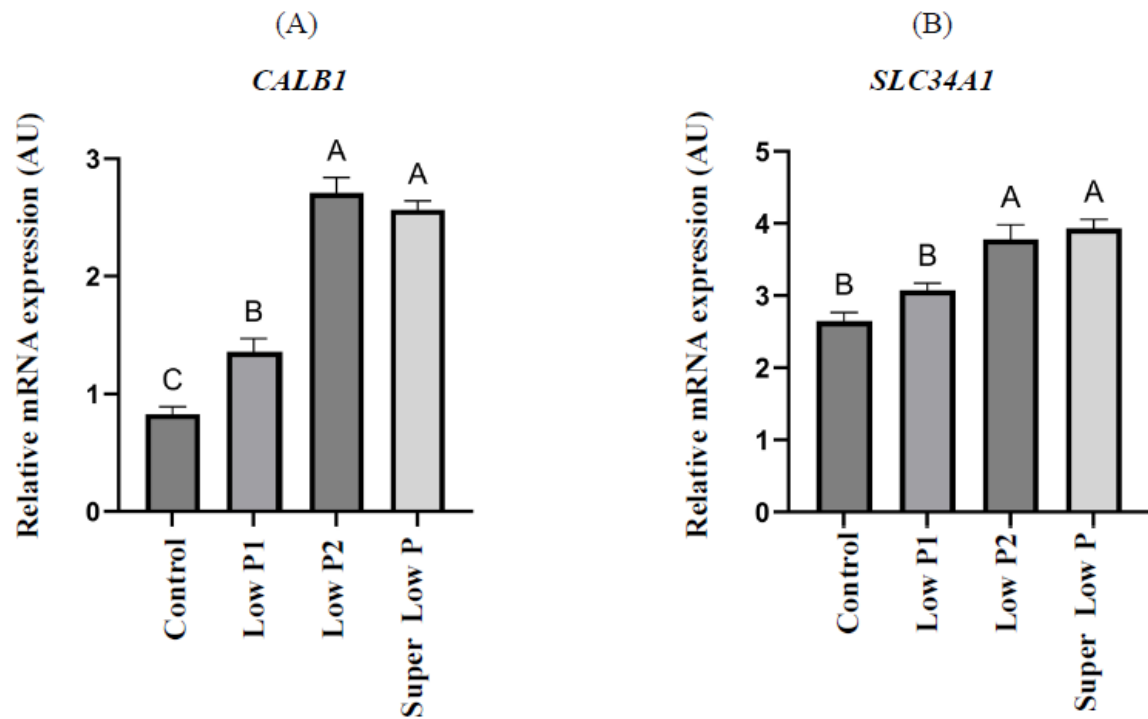
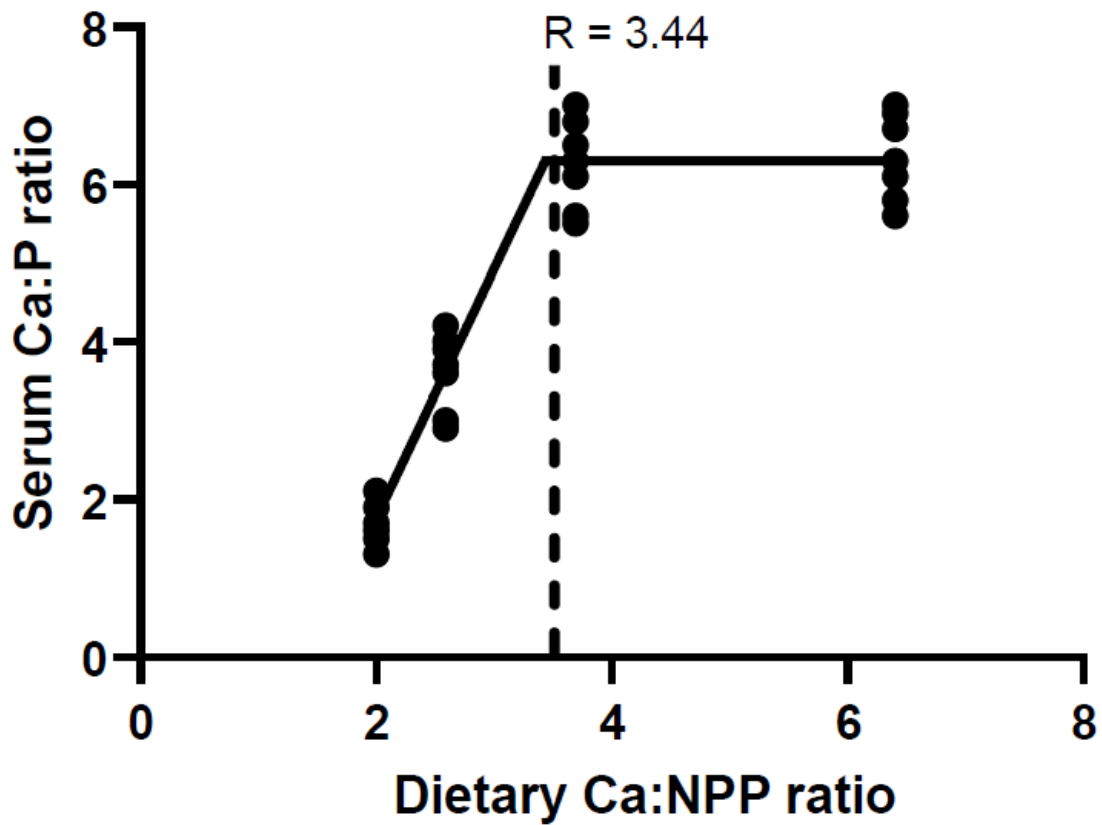


Fig. 4. mRNA expression levels of Calbindin-D28k (A, *CALB1*) and NaPi-IIa (B, *SLC34A1*) in the kidney. Values are means $\pm$ SEM (n = 7). Bars lacking a common superscript letter differ significantly ($p < 0.05$). The levels are represented as arbitrary units (AU).

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464 **Fig. 5.**Relationship between serum calcium (Ca):phosphorus (P)ratio and dietary Ca:non-phytate
465 phosphorus (NPP)ratio. The serum Ca:P ratio exhibits an upward trend associated with the stepwise
466 increase in dietary Ca:NPPratio. This response appears to reach a plateau as the dietary ratio exceeds
467 the estimated threshold of $R = 3.44$ ($R^2 = 0.9475$).

