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Effect of substituion of different levels of nano dicalcium phosphate ($CaHPO_4$) with dicalcium phosphate on serum blood biochemical parameters, intestinal morphology and microflora and bone characteristics in broiler Japanese quails

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Abstract: The aim of the current study was to determine the effect of substituion of different levels of nano dicalcium phosphate (NDCP) with dicalcium phosphate (DCP) in quails dietary regims on serum blood biochemical parameters, intestinal morphology and microflora and bone characteristics in broiler Japanese quails. A total of 360 quails were used in experimental randomized design from with 6 treatments of DCP and NDCP (60, 80 and 100 percetnage) and five replicates with 12 quails perv each replicate. The experimental period lasted 35 days. At the end of the study period blood samples were taken to determine serum blood bichemical and liver enzemye activities. For intestinal morphology determination the sample tissue of midway between jejunum and ileum were collected and samples of digesta from ileum were taken for microbial contents assessment. Bone characteristics such as tibia ash, phosphorus, calcium, and the ratio of tibia weight to length left tibia were analyzed. Results showed significant differences in serum Alanine transaminase (ALT), Aspartate transaminase (AST), and phosphorus levels among the study groups ($p \leq 0.05$). Additionally, there were significant differences in tibia ash content, phosphorus and calcium levels, weight to length ratio, and tibia weight ($p \leq 0.05$). The lowest villus height and width ratio was observed in the 60% dicalcium phosphate treatment, and the lowest crypt depth was found in the 100% dicalcium phosphate treatment. Data also showed that there were significant effect of dietray NDCP for intestinal microflora in experimental quails ($p \leq 0.05$). Overall, we could conclude that replacing standard dicalcium phosphate with nano dicalcium phosphate did not negatively impact the health of the quails and safely enhanced bone growth through nano mineral supplementation.

Keywords: Nano dicalcium phosphate, Japanese quail, Blood serum biochemical parameters, Intestinal morphology, Bone characteristics.

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Introduction

Calcium and phosphorus are vital nutrients for the growth and development of body tissues and organs, from early stages of growth to periods of rearing and egg production (STANQUEVIS et al., 2021). These two elements work synergistically, complementing each other to play essential roles in lipid and protein transport processes within the body (BROOKS et al., 2013). Key functions of calcium and phosphorus include the synthesis of phospholipids, collagen fibers, energy transfer, nerve signaling, and various metabolic reactions (BORDA-MOLINA et al., 2020). Dicalcium phosphate, produced from the reaction between phosphoric acid and calcium carbonate, serves as the primary source of dietary phosphorus and calcium for poultry (MANN et al., 2015). Additionally, phosphorus is fundamental in blood pH regulation and buffering systems, as well as in the formation of phospholipids, which facilitate fatty acid transport throughout the body (METSON et al., 2012).

Nanotechnology in nutrition has the potential to alter the physical structure of nutrients without modifying their chemical composition, thereby enhancing bioavailability. One of the critical features of nano-sized particles is their increased surface-area-to-volume ratio, which can significantly improve absorption. For instance, studies have shown up to a 40-percentage increase in nutrient absorption in mice when nanoscale particles are used (WEI et al., 2020).

Nanotechnology is a new emerging era of quail's nutrition and becoming a popular trend because of its versatile advantages like increased bioavailability and increasing absorption with reduction in excreta and its minimum quantity required in feed compared to conventional source (SWAIN et al., 2015). Nano particles are synthesized under laboratory conditions through chemical processes and specialized equipment. They also may be usefull to fulfil the requirement of major and micro elements to promote growth rate, enhance the feed efficiency and overall performance for quails by enhanced bioavailability. Dicalcium phosphate is commonly provided in processed mineral salt forms for poultry production (Marchiori et al., 2019), but under laboratory conditions, it can be converted into nanoscale particles using ultrasonic waves. Nano dicalcium phosphate, due to its exceptionally small particle size and high surface area, can facilitate the transfer and absorption of calcium and phosphorus ions across cell membranes (LABHASETWAR et al., 2008).

The result of (Vijayakumar and Balakrishnan, 2014) study has been showed that the feeding of calcium and phosphorus in nano particle form instead of using conventional di-calcium phosphate has increased the feed efficiency in the broiler birds. Some researchers found that silver particles could bind to ion-transport proteins in the intestines, potentially blocking ion absorption channels and reducing the uptake of essential nutrients such as iron, calcium, and potassium. The study suggested nanoparticle technology as a solution to enhance nutrient absorption by preventing these interactions (OGNIK et al., 2017).

An attempt has been made in the current study to determine usage of nano dicalcium phosphate as a primary dietary source of phosphorus and calcium and evaluation the biological effects of replacing standard dicalcium phosphate with nano dicalcium phosphate on serum biochemical parameters, intestinal morphology, and bone characteristics were then evaluated in Japanese quails.

Materials and methods

At the beginning of the experiment, total opf 360 one days old broiler quails were weighed collectively, and based on the obtained weights, they were divided into six group treatmennts and five replicate with 12 quails each with uniform average weights across the treatments. The experimental treatments included 100%, 80% and 60% NDCP substituation with same Ingredients of DCP respectively. The nutritional items of the experimental diets were determined according to the AOAC (2005) and nutritional composition of the quail diets was formulated according to the NRC 1994 nutrient requirements for Japanese quails as shown in Table 1.

Table 1. Ingredients and nutrient composition of experimental diets for experimental Japanese quails

Ingredients (%)	DCP 100%	DCP 80%	DCP 60%	NDCP 100%	NDCP 80%	NDCP 60%
Corn grain	49.01	49.01	49.01	49.01	49.01	49.01
Soybean meal	45.08	45.08	45.08	45.08	45.08	45.08
Vegetable oil	2.82	2.82	2.82	2.82	2.82	2.82
Methionine (DL)	0.2	0.2	0.2	0.2	0.2	0.2
Lysine (L)	0.1	0.1	0.1	0.1	0.1	0.1
Threonine	0.1	0.1	0.1	0.1	0.1	0.1
Dicalcium Phosphate (DCP)	1.39	1.11	0.83	0	0	0
Nano Dicalcium Phosphate (NDCP)	0	0	0	1.39	1.11	0.83
Oyster shell (Calcium Carbonate)	0.95	0.95	0.95	0.95	0.95	0.95
NaCl	0.36	0.36	0.36	0.36	0.36	0.36
Vitamin and Mineral Permix**	0.50	0.50	0.50	0.50	0.50	0.50
Grit	0	0.28	0.56	0	0.28	0.56
Nutrients composition						
Energy (Kcal/kg)	2900	2900	2900	2900	2900	2900
Crude Protein (%)	24	24	24	24	24	24
Crude Fiber (%)	4.23	4.23	4.23	4.23	4.23	4.23
Methionine (%)	0.53	0.53	0.53	0.53	0.53	0.53
Methionine + Cysteine (%)	0.65	0.65	0.65	0.65	0.65	0.65
Lysine (%)	1.2	1.2	1.2	1.2	1.2	1.2
Threonine (%)	0.95	0.95	0.95	0.95	0.95	0.95
Calcium (%)	0.80	0.80	0.80	0.80	0.80	0.80
Phosphorus (%)	0.30	0.30	0.30	0.30	0.30	0.30
Sodium (%)	0.15	0.15	0.15	0.15	0.15	0.15

**Vitamin Supplement Composition (per kg): 9,000,000 IU Vitamin A, 4,000,000 IU Vitamin D, 63,000 IU Vitamin E, 2.3 g Vitamin K, 2.6 g Thiamine, 6.5 g Riboflavin, 15 g Pantothenic Acid, 48 g Niacin, 3 g Pyridoxine, 1.7 g Folic Acid, 0.13 g Cobalamin.Mineral Supplement Composition (per kg): 105 g Manganese Oxide, 126 g Zinc Oxide, 16 g Copper Sulfate, 1.3 g Calcium Iodate, 0.038 g Selenium Premix.

Nano dicalcium phosphate preparation

The dicalcium phosphate used in this experiment was obtained from Pars Chemistry Company, under veterinary number D.C.P 47-87. A 40-gram homogenous sample of dicalcium phosphate was sent to the laboratory, where its phosphorus and calcium content was analyzed according to Iranian national standards ISIR 2513 and ISIR 513.

The dicalcium phosphate used contained 18.58% phosphorus and 22.17% calcium. Nano-sizing of dicalcium phosphate was conducted at the Nanotechnology and Advanced Materials Research Center at Isfahan University of Technology, where nano dicalcium phosphate particles smaller than 100 nanometers were produced using ultrasonic equipment (Fig.1).

Fig.1. Dicalcium phosphate and prepared dicalcium phosphate nano particles ($CaHPO_4$)

Blood samples collection and analaysis.

Blood samples were collected at the end of experimental period during slaughter, and were sent to the laboratory for biochemical analysis. After collection, the blood samples were stored below freezing, transported to the lab, and centrifuged. Blood serum was analyzed for calcium, phosphorus, albumin, total protein, globulin, cholesterol, and triglycerides using commercial biochemical kits, while liver enzyme concentrations were measured with laboratory kits.

Histomorphology of the intestine determination

For histomorphological analysis, approximately one centimeter of tissue was excised from the jejunum (midway between the bile duct entry and the Meckel's diverticulum) and the ileum (before the ileocecal junction). The samples were fixed in a 4% formalin solution and then prepared for microscopic examination by making cross-sections with a microtome (Chen et al., 2016).

Microbial flora of intestine evaluation

The microbial contents of the ileum for E. coli, Lactobacillus and Salmonella enterica were assessment by use one g of collected intestinal digesta samples and diluted with sterile 0.9% NaCl to 10 folds. The MRS agar LAB and nutrient media were used to perform the enumerations of LAB, E. coli and Salmonella enterica, respectively. For LAB and Salmonella enterica, the plates were incubated in anaerobic jars for 48 h at

30°C and 37 °C for E. coli (Kareem et al.,2016). The MRS1-Agar was used to determine Lactobacillus for 72h incubation at 37°C. After incubation, the bacteria were counted in petri dishes, the number of bacteria in the initial volume was calculated, and the counts were reported as $\log^{10}$ CFU per each g of digesta (Kheiri et al., 2024).

Bone characteristics assessment

On the last day of the experiment,after slaughter the left tibia was removed from each quail, and fat was extracted with ether in a Soxhlet apparatus. The bones were then placed in water at 60°C for 24 hours to dry and reach a stable weight. After drying, the bones were ground and incinerated in a furnace at 600°C for 8 hours to obtain ash. Calcium and phosphorus content in the bone ash was determined by titration with hydrochloric acid. Bone properties, including weight, length, width, and relative ash percentage, were measured. Bone length was determined using a caliper with 0.01 mm precision, measuring the distance between the two ends of the bone. For ash percentage, the bones were dried in an oven at 100°C for 24 hours, ground, placed in a crucible, and incinerated at 600°C to measure the ash content.

Statistical analysis

The collected data were analyzed using Excel and SPSS software version 26 and the comparisons between treatment means were conducted with Tukey's test at a 5% probability level ($p \leq 0.05$).

Results

Blood serum biochemical parameters and liver enzymes activities

The results of the analysis of variance and Duncan's post-hoc test at the 5% level for the biochemical serum parameters and liver enzymes at 35 days of age in Japanese quails are presented in Table 3. The results of this experiment as shown in table 2 showed significant differences between the groups regarding the use of dicalcium phosphate and nano dicalcium phosphate in the levels of ALT, AST, and total blood phosphorus ($p < 0.05$). However, no significant differences were observed in the values of other parameters tested.

Table 2. Effect of experimental treatments on blood serum biochemical and liver enzyme activities of Japanese quail

Treatments	ALB (g/dl)	ALT (U/l)	AST (U/l)	TG (mg/dl)	TP (g/dl)	Ca (mg/dl)	P (mg/dl)	Chol (mg/dl)	Glu (g/dl)
DCP 100%	1.44	3.26^{ab**}	221.0^{ab}	188.2	3.74	11.62	9.78^{ab}	196.4	2.29
DCP 80%	1.27	3.80^{ab}	243.2^{b}	171.6	3.10	12.64	8.94^{a}	195.6	1.82
DCP 60%	1.54	3.86^{b}	241.8^{b}	200.2	3.26	12.42	8.56^{a}	218.8	1.71
NDCP100%	1.33	3.04^{a}	209.6^{a}	194.4	3.38	12.08	12.26^{c}	170.2	2.05
NDCP 80%	1.29	3.68^{ab}	235.0^{ab}	186.0	3.32	13.36	10.90^{bc}	227.8	2.02
NDCP 60%	1.35	3.76^{ab}	232.2^{ab}	182.2	3.22	12.08	9.20^{a}	211.8	1.86
P.Value	n.s	**	**	n.s	n.s	n.s	**	n.s	n.s

**Means without similar letters show significant differences in each columns ($P \leq 0.05$).n.s: Not significant. ALB: Albumen, ALT: Alanine transaminase, AST: Aspartate transaminase,TG: Triglyceride,TP: Total blood phosphorus,Ca:Blood calcium,P: Phosphorous,Chol: Blood serum cholesterol, Glu: Blood serum glocuse.

Bone characteristics of quails

The results of the analysis of variance and Duncan's post-hoc test at the 5% level for the bone characteristics of Japanese quails at 35 days of age are presented in Table 3. The results revealed significant differences between the groups concerning the use of dicalcium phosphate and nano dicalcium phosphate in the amount of tibia ash, phosphorus, calcium, and the ratio of tibia weight to length ($p < 0.05$). However, no significant differences were observed in the tibia length, tibia width, and the ratio of width to length.

Table 3. Effect of experimental treatments on bone characteristics of Japanese quail

Treatments	Tibia Weight (mg)	Tibia Length (mg)	Tibia Width (mg)	Weight/ Length Ratio	Width/ Length Ratio	Tibia Ash (%)	Calcium (%)	Phosphorus (%)
DCP 100%	757.80^{a**}	56.02	2.98	13.54^{a}	5.32	50.86 ^{a}	16.50^{ab}	9.34^{ab}
DCP 80%	765.20^{a}	53.82	3.11	14.22^{ab}	5.78	50.82 ^{a}	16.14^{a}	9.17^{b}
DCP 60%	729.00^{a}	55.22	2.95	13.22^{a}	5.36	49.90 ^{a}	15.64^{a}	8.36^{a}
NDCP 100%	855.40^{b}	54.46	3.08	15.76^{c}	5.68	52.64 ^{b}	18.28^{c}	10.48^{d}
NDCP 80%	833.00^{b}	55.00	3.04	14.96^{bc}	5.53	52.78 ^{b}	17.66^{bc}	9.77^{c}
NDCP 60%	755.20^{a}	56.04	3.02	13.49^{a}	5.41	51.64 ^{ab}	16.86^{ab}	9.28^{b}
P.Value	**	n.s	n.s	**	n.s	**	**	**

**Means without similar letters show significant differences in each column (P≤ 0.05). n.s: Not significant.

Morphology of the intestine tissue

The effect of different treatments on the morphological parameters of the jejunum is presented in table 4. The results showed that the highest values for villus height and villus width were observed in the groups supplemented with 100% nano dicalcium phosphate.

The deepest crypt depth was observed in the 80% nano dicalcium phosphate treatment group. Additionally, the lowest values for villus height and width were found in the 60% dicalcium phosphate treatment, and the lowest crypt depth was found in the 100% dicalcium phosphate group.

Table 4. Effect of experimental treatments on intestine morphology of Japanese quail

Treatment	Villus height (µm)	Villus width (µm)	Crypt depth (µm)
DCP 100%	548^{ab**}	73.2^{ab}	54.2
DCP 80%	562^{ab}	81.0^{abc}	59.8
DCP 60%	476^{a}	70.6^{a}	64.6
NDCP 100%	626^{b}	92.4^{c}	65.6
NDCP 80%	565^{ab}	85.8^{bc}	68.2
NDCP 60%	555^{ab}	80.0^{abc}	59.4
P.Value	**	**	n.s

**Means without similar letters show significant differences in each columns (P≤ 0.05). n.s: Not significant.

Intestinal microflora

The effect of different treatments on the microbila parameters of the ileum is presented in table 5. The results revealed showed that Lactobacillus clonies were increased significantly and E-coli and Salmonella enterica clonies were decreased instead in the groups supplemented with 100,80 and 60 % of NDCP with DCP.

Table 5. Effect of experimental treatments on intestine microflora of Japanese quail

Treatment	E-coli (Cfu/g)	Lactobacillus (Cfu/g)	Salmonella enterica (Cfu/g)
DCP 100%	4.28[c]	5.58[a]	5.70[c]
DCP 80%	4.39[b]	5.35[b]	5.77[b]
DCP 60%	4.62[a]	5.11[c]	5.85[a]
NDCP 100%	3.98[c]	5.94[a]	5.68[c]
NDCP 80%	4.11[b]	5.69[b]	5.72[b]
NDCP 60%	4.35[a]	5.46[c]	5.76[a]
P.Value	**	**	**

**Means without similar letters show significant differences in each column ($P \leq 0.05$).

Discussion

Blood biochemical variables are actually indicators of instability, and their changes can be influenced by both internal and external factors, such as the animal's diet. Various factors affect their levels (CIURESCU et al., 2020). The results from this experiment indicated that there were significant differences in liver enzymes ALT, AST and blood phosphorus levels between the study groups using dicalcium phosphate and nano-dicalcium phosphate ($p < 0.05$). An increase in phosphorus intake from the diet showed a direct correlation with an increase in blood phosphorus levels. Regarding the liver enzymes ALT and AST, the groups treated with 100% nano-dicalcium phosphate showed values of 209.60 and 3.04, respectively, with the lowest AST value observed in the group using 100% nano-dicalcium phosphate. Additionally, a significant difference in blood phosphorus levels was found between the treatments of 60% dicalcium phosphate and nano-dicalcium phosphate (MAKOLA et al., 2021). It was reported that the use of nano-dicalcium phosphate in broiler chicken diets significantly altered liver enzymes. As phosphorus absorption from the digestive tract increased, AST levels rose, while ALT levels in the plasma decreased (NOURMOHAMMADI et al., 2011; DE SOUSA et al., 2015).

In this study, blood calcium levels were not influenced by different phosphorus or nano-dicalcium phosphate levels. Previous studies on broiler chickens have shown that blood calcium levels are unaffected by the amount of phosphorus absorbed from the diet (De Sousa et al., 2015), which aligns with the results obtained in this research. The lowest blood phosphorus level in this study was associated with the 60% dicalcium phosphate treatment, while the highest blood phosphorus level was observed in the 100% nano-dicalcium phosphate treatment, demonstrating the relationship between dietary phosphorus content and blood phosphorus levels. Studies have shown that reducing phosphorus particle size increases phosphorus absorption (MAKOLA et al., 2021).

The highest blood phosphorus level in this study was seen in the 100% nano-dicalcium phosphate group, which suggests better phosphorus absorption in its nano form. In this experiment, blood cholesterol levels did not show any significant effect between the experimental treatments. With increased phosphorus intake, plasma phosphorus levels also increased, but there were no significant differences observed in HDL, triglycerides, calcium, total protein, or glucose levels. These findings are consistent with previous studies on the use of nano-dicalcium phosphate in broiler diets (Poinern et al., 2009; Vijayakumar et al., 2015). Moreover, Vijayakumar et al. (2014, 2015) found that the use of calcium phosphate nanoparticles did not significantly affect hemoglobin levels, total red blood cell count, total white blood cell count, or differential white blood cell count compared to the control group fed regular dicalcium phosphate. They also reported that replacing regular dicalcium phosphate with nano-calcium phosphate did not affect bird health. In another study, Poinern et al. (2009) demonstrated that reducing particle size in animal feed increases nutrient absorption, without any negative effects such as anemia, infection, or other adverse outcomes in birds. The morphological analysis of the intestines showed that the highest values for villus height and villus width were observed in the treatments supplemented with 100% nano-dicalcium phosphate. Although there was no statistically significant difference in crypt depth between the groups, the highest values were found in the 80% nano-dicalcium phosphate treatment. The increase in villus length and width in this study can be attributed to the improved absorption and utilization of the specific nano-sized particles. Increased surface area and absorption capacity are associated with longer villi, leading to the maturation of enterocytes at the villus tips, which is similar to the results reported by Al-Beitawi et al. (2017). in the use of nanoparticles in broiler diets.

Ciurescu et al. (2020) found that poor nutrient absorption and digestion were associated with shorter villi, resulting in decreased absorption levels and fewer mature enterocytes. Makola et al. (2021) demonstrated that the use of nano-dicalcium phosphate in broiler diets improved villus length and mineral absorption, which aligns with the findings of this study. Both phosphorus and calcium have received considerable attention from researchers due to their critical roles in bone formation (STANQUEVIS et al., 2021).

The results of the bone characteristics analysis in this experiment revealed significant differences in the ash content, phosphorus, calcium, weight-to-length ratio, and weight of the tibia ($p < 0.05$). The highest levels of phosphorus, calcium, weight-to-length ratio, and tibia weight were observed in the group treated with 100% nano-dicalcium phosphate.

The lowest phosphorus content in the bones was found in the third treatment, the 60% dicalcium phosphate group, which may reflect the correlation between dietary phosphorus levels and phosphorus storage in the bones. El-Sheikh et al. (2018) showed that the use of nano-dicalcium phosphate in the diets of laying hens improved calcium and phosphorus absorption, as well as increasing eggshell thickness.

This experiment also found significant differences in tibia ash content, phosphorus, calcium, weight-to-length ratio, and tibia weight, while no significant differences were observed in tibia length, width, or width-to-length ratio. Matuszewski et al. (2020) reported that the use of nano-dicalcium phosphate in broiler diets improved bone characteristics.

David et al. (2021) found that reducing dietary phosphorus levels, even without a significant effect on animal performance, led to a significant decrease in bone ash content, which is consistent with the results of this experiment. As phosphorus levels in the bones increased, so did the ash content of the bones. The highest bone ash content in this study was observed in the fourth treatment (100% nano-dicalcium phosphate), likely due to better digestion and absorption of phosphorus and calcium through the nano-forms of dicalcium phosphate.

Ciurescu et al. (2021) stated that higher phosphorus absorption directly improves bone characteristics. Nuamah et al. (2024) also reported that increasing phosphorus absorption from the diet in the intestines leads to higher blood phosphorus levels and an increase in bone phosphorus content. Regarding tibia weight, this experiment observed an increase in tibia weight in line with the use of nano-dicalcium phosphate. Previous studies have shown that dicalcium phosphate affects the bone characteristics of broiler chickens, with higher absorption of dicalcium phosphate resulting in greater bone length and width, higher bone strength, and increased bone ash content, which aligns with the findings of this experiment (CIURESCU et al., 2023; VIJAYAKUMAR et al., 2014).

Conclusion

The observed increase in villus length and width in this study can be attributed to the enhanced absorption and bioavailability properties associated with the nano-scale particle size. Nano-dicalcium phosphate not only improves the bioavailability of calcium and phosphorus but also facilitates their absorption within the gastrointestinal tract, effects that are likely due to the exceptionally small particle size and expanded surface area available for absorption.

Supplementation with nano-calcium phosphate, as opposed to conventional dicalcium phosphate, demonstrated no adverse impacts on avian health and effectively supported bone growth, underscoring the potential benefits of safe, nano-mineral supplementation.

Ethics approval

The current reasearch were done at quail's research farm of Agriculture Faculty of Islamic Azad Univeristy Khorasgan Branch and all of the procedures includes experimental plan, sampling of data, slaughter was done according to the animal wellness and welfare local committee of Islamic Azad University of Khorasgan Branch, Isafahan, Iran (Approval reference code number: 2020.05.08).

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Conflict of interests

The authors had no conflict of interests in the current study.

Data availability: All data presented in this study will available free of charge for any researcher upon reasonable request from the corresponding author.

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