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Assessment of Calcium Phosphate Nanoparticles as Safe Mineral Supplement for Broiler Chicken

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Abstract

An experiment was carried out with the objective with assessment on the safety of calcium phosphate nanoparticles as mineral supplement to broiler chicken. With the help of an indigenous laboratory equipment calcium phosphate nanoparticles were prepared by wet chemical method. Calcium phosphate nanoparticles were analyzed for its size and shape using TEM. It was ranged from 20 to 90 nm with spherical shape. The calcium and phosphorous content was estimated. Results show that 30.81 per cent calcium and 15.48 per cent phosphorus. Using the *Vero* cell line *in vitro* cytotoxicity study carried out that indicated 10 mg/ml of calcium phosphate nanoparticles did not provoke observable cellular insult. The *in vivo* assessment was also done using hematological and serum biochemistry parameters in birds fed with calcium phosphate nanoparticles at graded concentration at 50, 60, 70, 80, 90 and 100% of the phosphorus content of coarse particles of dicalcium phosphate in the diet from day old to 28 days. The results indicated that Calcium phosphate nanoparticles did not exert any significant ($P<0.05$) influence on haemoglobin, Packed cell volume, total erythrocytes count, total leucocytes count, and differential count of WBC compared to control (fed with dicalcium phosphate). The inclusion of calcium phosphate nanoparticles from 50 to 100% did not have any significant negative influence on the serum glucose, total protein, albumin, triglyceride, cholesterol, serum urea, creatinine, serum Aspartate amino Transferase (AST), Alanine amino Transferase (ALT) and Alkaline Phosphatase (ALP). Hence, it was concluded that calcium phosphate nanoparticles are safe to use since an adult bird would consume at a concentration not more than 6.4 mg/ml which is well within the safe level of 10mg/mlas identified by *in vitro* cytotoxicity test on *vero* cell line.

Keywords: Biochemistry, Calcium Phosphate Nanoparticles, Hematology, *In vitro*Cytotoxicity, Serum, TEM, *Vero* Cell Line

1. Introduction

Nanotechnology inspite of its benefits also introduces a new order of health risks. Greater chemical reactivity and bioavailability of nanomaterials might result in greater toxicity of nanoparticles compared to the same unit of mass of larger particles of the same chemical composition[1,2]. Though calcium phosphate nanoparticles are used as adjuvant in drug delivery, its effect on oral intake has not been evaluated. The supplement of calcium and phosphorus in the form of nanoparticles is considered a strategy to bring down the cost of calcium and phosphorus supplement and the feed[3]. Hence, such a nanoparticles based strategy needs to be explored. However, nanoparticle behave differently from coarse particles and therefore its usage as mineral supplement is viewed skeptically. Hence it is imperative to verify its safety on biological medium. *In vitro* cytotoxicity assessments are often used to establish the safety of any nutrient. In this context, this research was focused on four aspects viz., production of calcium phosphate nanoparticles, confirmation of the size and morphology of calcium phosphate nanoparticles and assessment to determine of calcium phosphate nanoparticles as a safe mineral supplement by *in vitro* cytotoxicity and confirmation by *in vivo* through assessing the hematological and blood biochemical parameters of broiler chicken fed for 28 days with graded level of calcium phosphate nanoparticles.

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2. Materials and Methods

The calcium phosphate nanoparticles were prepared by following the procedure outlined by[4]. Wherein an aqueous solution of calcium nitrate (18mM) and aqueous solution of diammonium hydrogen phosphate (10.8mM) were mixed after the pH of both the solutions was adjusted to 9 using 0.1M sodium hydroxide. The mixture of solutions were centrifuged at 12000 rpm and the particles were allowed to settle at the bottom which was then spread out as a thin film in trays and dried in hot air oven at 90°C. After drying white soft powder (calcium phosphate nanoparticles) was obtained and subjected to further analysis.

An indigenous laboratory model for preparing calcium phosphate nanoparticles was fabricated. The unit had two plastic tanks of capacity 15 litres each. In one tank calcium nitrate solution (14 L) and in the other tank diammonium hydrogen phosphate solution (14 L) was taken. Each tank had an outlet at the bottom, which was fitted with narrow plastic tubes. Each of the outlet tubes were then connected to an 18 G injection needle. The solutions were allowed to flow out through the 18 G injection needle and fall into a beaker. The beaker was placed over a magnetic stirrer which causing continuous mixing of the solutions. This indigenous laboratory model was used for preparing two kilogram of calcium phosphate nanoparticles required for the study.

In prepared calcium phosphate nanoparticles six samples were screened for the calcium and phosphorus contents using Atomic Absorption Spectrophotometer (AAS) (Perkin-Elmer, Model 3110, 1994) (procedure outlined in the AAS reference manual) and calorimetric method[5] respectively.

By adopting the procedure of[6], using Transmission Electron Microscopy (TEM) the size and morphology of the prepared calcium phosphate nanoparticles was carried out.

One drop of one per cent phosphotungstic acid was mixed with two drops of aqueous dispersion of nanoparticles on a para film using a micropipette. A copper grid was placed over the surface of the liquids and left for two minutes. The copper grid was lifted and the excess fluid was absorbed using a tissue paper. The copper grid was air dried in an incubator at 37°C. The dried copper grid was then examined under a transmission electron microscope (Tecnai 10, Philips operated under 80 KVA pressure). The morphology of identified calcium phosphate nanoparticles was studied and their size measured.

In order to determine safety of calcium phosphate nanoparticles *in vitro* experiment on its effect on *vero* cell line was assessed.

The African green monkey kidney cell line (*vero*), obtained from Department of Animal Biotechnology, Madras Veterinary College was used for cytotoxicity study. The *vero* cell lines were maintained in T 25cm^2 flask (Nunc, USA) using 10% fetal bovine serum and Dulbecco's Modified Eagle Medium (DMEM) at 37°C with 5% CO_2 in CO_2 incubator (New Brunswick, Eppendrorfcalaxy 170 S).

Once monolayer was formed after 24 hours of incubation, the spent medium was discarded. The cells were trypsinised using 0.25 % Trypsin Versene Glucose (TVG) solution and were seeded in twelve well plates (Nunc, USA). The cell line was further incubated in a CO_2 incubator at 37° C with 5% CO_2 until a monolayer formed. To this monolayer, an a liquot of 1 ml containing 10, 50 and 100 mg of calcium phosphate nanoparticles were added in triplicate and then incubated along with control with only maintenance medium for further 24 hours. At the end of incubation, the cells were viewed under phase contrast inverted microscope (Nikon Eclipse TS 100).

Confirmation test was carried out in seventy day old male broiler chicks (cobb-400) belonging to a single hatch and distributed randomly to the seven experimental groups of 10 chicks each. Calcium phosphate nanoparticles was fed to birds at 50, 60, 70, 80, 90 and 100% of the phosphorus content of coarse particles of dicalcium phosphate in the diet from a day old to 28 days. Control birds were fed phosphorus from dicalcium phosphate. Blood samples were collected from the birds at the end of 28 days. About 0.5 ml of blood was used to estimate haemoglobin (Hb) concentration as per the method of Sahli's Acid Hematin[7], Packed Cell Volume (PCV) using wintrobe's microhematocrit method[8], Total Erythrocytes Count (TEC) and Total Leucocytes Count (TLC) by using Nambiar's diluting fluid[9], Differential Count (DC) by using modified Leishman-Giemsa stain as per the method described by[9].

The serum glucose was estimated by glucose oxidase method. The total protein and albumin levels in the serum samples were estimated based on direct biuret method. Serum Triglyceride was estimated based on glyceride glycerol analysis and cholesterol was estimated based on cholesterol dehydrogenase/peroxidase method. The serum urea levels were estimated based on glutamate dehydrogenase method. Serum creatinine was estimated based on Jaffes method. Serum Aspartate amino Transferase

(AST), Alanine amino Transferase (ALT) and Alkaline Phosphatase (ALP) were estimated by IFCC (International federation of clinical chemistry) method[10]. All the estimations were carried out in Serum auto analyzer (Biosystems 320) using commercial reagent kit.

3. Results and Discussions

The indigenous laboratory model for preparing calcium phosphate nanoparticles was able to produce 2 g calcium phosphate nanoparticles per hour. The mineral analysis of six samples of calcium phosphate nanoparticles indicated that it contained 30.81 per cent of calcium and 15.48 per cent of phosphorus. The ratio of calcium to phosphorus was 1.99:1 as against 1.39:1 in Dicalcium phosphate. This desirable ratio of 2:1 is conducive to formulate mineral mixture as the volume required to achieve calcium and phosphorus using calcium phosphate nanoparticles is less compared to using dicalcium phosphate. Thus incorporating calcium phosphate nanoparticles in mineral mixture paves more space to accommodate other minerals. Further the requirement of both calcium and phosphorus in poultry mineral mixture can be attained by using single ingredient viz., calcium phosphate nanoparticles as against two ingredients viz., Dicalcium phosphate and Calcite.

White soft powders of calcium phosphate nanoparticles were produced. When viewed through Transmission Electron Microscopy (TEM) calcium phosphate nanoparticles were well dispersed and measuring between 20 and 90 nanometers with spherical shape. Calcium phosphate nanoparticles in the range of 30-40 nm diameter was produced by[6]. Calcium phosphate nanoparticles had spherical morphology with a diameter of 10-20 nm and 40-50 nm respectivelyby[4,11]. The spherical morphology of calcium phosphate nanoparticles is desirable from nutrition point of view as sphere will always have relatively larger surface area compared to other morphologies. It is also postulated that nanoparticles supplementation results in higher availability of the respective element. This could be attributed to the smaller particle size and suggests that supplementation in nanoparticles size can enhance absorption. This supports the findings of past studies that the extent of particle uptake is inversely proportional to the particle size[12]. Also indicated that cell uptake depends on the nanoparticles size, and smaller particlespossess greater uptake in general[13].

Vero cell line at the concentration of 10^6cells/ml containing twelve well plates were used for carry out *in vitro* cytotoxicity assay for the produced calcium phosphate nanoparticles concentration of 10, 50 and 100 mg for one ml of distilled water. The control wells containing maintenance medium with this the appearance of the cells were compared and it was observed that *vero* cells were intact at 10 mg concentration of calcium phosphate nanoparticles per ml. It means that at 10 mg/ml was not toxic to cells. Rounding and focal detachment of *vero* cell line changes were observed at 50 and 100 mg/ml levels conclude that at 50 and 100 mg/ml, the calcium phosphate nanoparticles were toxic to the cells.

Water intake of the birds was two times of their feed ingestion. If 100 g of feed is ingested per bird along with 200 ml of water, the quantity of calcium phosphate nanoparticles will be only 6.4 mg/ml. The birds in this T6 group would have consumed 1.9 g of calcium phosphate nanoparticles in 100 g of feed. If we calculate this 1.9 g of calcium phosphate nanoparticles in 100 g of feed was mixed with 200 ml of water that bird would drink with related to feed ingestion, the final concentration will be 1/3 of 1.9 g i.e. 6.4 mg/ml only. This was less than the safe zone of 10 mg/ml, resulted in the cell line study.

The microscopic picture of vero cell line subjected to various concentrations of calcium phosphate nanoparticles is shown here under (Figure 1).

And reported that if concentration of silver nanoparticles were increased then damage to the cell line also increases[14,15].

The effect of calcium phosphate nanoparticles on hematological parameters of broilers is presented in Table 1. Birds fed with calcium phosphate nanoparticles at graded concentration at 50, 60, 70, 80, 90 and 100% of the phosphorus content of coarse particles of dicalacium phosphate in the diet from day old to 28 days. The results indicated that Calcium phosphate nanoparticles did not

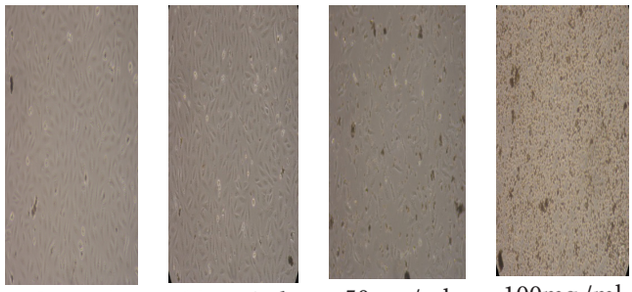

Figure 1. Effect calcium phosphate nanoparticles on *vero* cell line at different concentrations (image- 100X).

Table 1. Effect of various levels of calcium phosphate nanoparticles on haematological (Mean*±SE) parameters of broilers at 4th week of age*

Treatment groups	Calcium phosphate nanoparticles (%)	Hb (g/dl)	PCV (%)	RBCx10⁶/mm³	WBCx 10³/mm³	Differential count (per cent)			
						Lymphocyte	Heterophil	Eosinophil	Monocytes
CONTROL	0	3.80 ±0.57	29.0 ±0.84	2.56 ±0.08	19.06 ±0.71	65.80 ±1.24	32.20 ±0.86	1.60 ±0.51	0.40±0.24
T1	50	3.62 ±0.06	28.60 ±0.51	2.55 ±0.17	18.98 ±0.94	65.40 ±1.03	32.80 ±0.66	1.20 ±0.37	0.60±0.40
T2	60	3.52 ±0.09	28.40 ±1.36	2.67 ±0.09	18.74 ±0.97	63.40 ±1.94	34.80 ±1.59	1.40 ±0.40	0.40±0.24
T3	70	3.83 ±0.01	28.20 ±1.16	2.61 ±0.03	19.00 ±1.00	65.40 ±1.36	33.40 ±0.81	0.60 ±0.40	0.60±0.40
T4	80	3.70 ±0.03	28.00 ±0.71	2.59 ±0.09	18.60 ±0.79	65.00 ±0.84	33.40 ±0.68	1.00 ±0.63	0.60±0.40
T5	90	3.54 ±0.08	29.00 ±1.00	2.54 ±0.08	18.50 ±0.94	65.40 ±0.93	33.00 ±1.00	0.80 ±0.49	0.80±0.37
T6	100	3.44 ±0.04	28.80 ±1.24	2.55 ±0.10	18.66 ±0.75	64.20 ±1.16	33.80 ±0.80	1.60 ±0.51	0.40±0.24

Mean of five observations
*: Non significant

exert any significant (P<0.05) influence on haemoglobin, Packed cell volume, total erythrocytes count, total leucocytes count, and differential count of WBC compared to control (fed with dicalcium phosphate)[16].

The effect of calcium phosphate nanoparticles on serum biochemical parameters of broilers is presented in Table 2. The inclusion of calcium phosphate nanoparticles from 50 to 100% did not have any significant negative influence on the serum glucose, total protein, albumin, cholesterol, serum urea, creatinine[17], triglyceride[18], serum Aspartate amino Transferase (AST), Alanine amino Transferase (ALT) and Alkaline Phosphatase (ALP)[19].

Hence, it was concluded that calcium phosphate nanoparticles are safe to use since an adult bird would consume at a concentration not more than 6.4 mg/ml which is well within the safe level of 10 mg/ml as identified by *in vitro* cytotoxicity test on *vero* cell line.

A non significant difference in the haemato logical parameters studied indicated that the intervention made do not affect the health of the experimental birds which reinforces the safety of mineral supplements through calcium phosphate nanoparticles instead of dicalcium phosphate.

4. Conclusion

Calcium phosphate nanoparticles contained 30.81 per cent of calcium and 15.48 per cent of phosphorus. The ratio of calcium to phosphorus was 1.99:1, which is desirable to formulate mineral mixture for broilers. The calcium phosphate nanoparticles were in the form of white soft powder, well dispersed when viewed through Transmission Electron Microscopy (TEM) and had spherical shape with diameter measuring between 20 and 90 nanometers The *in vitro* cytotoxicity test on *vero* cell line revealed that at10 mg concentration of calcium phosphate nanoparticles per ml, the *vero* cells were intact which indicate that at 10 mg/ml was not toxic to cells. Since an adult bird will not consume at concentration more than 6.4 mg/ml, it is concluded that Calcium phosphate nanoparticles can be safely used as mineral supplement. The hematological and biochemical parameters of broiler birds fed with graded level of Calcium phosphate nanoparticles from 50% to 100% replacing phosphorus content of coarse particles of dicalcium phosphate also confirmed that calcium phosphate nanoparticles do not cause any adverse effect on the health of broilers.

Table 2. Effect of various levels of calcium phosphate nanoparticles on serum biochemical (Mean*±SE) parameters of broilers at 4th week of age*

Treatment groups	Inclusion level of Calcium phosphate nanoparticles (%)	Glucose (mg/dl)	Total Protein (g/dl)	Albumin (g/dl)	Triglyceride (mg/dl	Cholesterol mg/dl)	Urea (mg/dl)	Creatinine (mg/dl)	AST (U/L)	ALT (U/L)	ALP (U/L)
CONTROL	0	163.40±6.58	3.32 ±0.07	2.28±0.06	78.00±5.25	134.60±7.51	27.48±0.36	0.33±0.02	234.60±5.51	2.10±0.84	2298.60±17.51
T1	50	169.00±6.79	3.58 ±0.30	2.35±0.08	71.40±8.76	132.20±9.97	27.36±0.63	0.31±0.01	232.40±5.97	2.30±0.52	2312.40±19.97
T2	60	161.40±9.00	3.19 ±0.25	2.04±0.07	73.80±9.56	138.40±9.95	25.98±0.34	0.34±0.02	238.40±6.95	2.00±0.45	2288.40±24.95
T3	70	162.20±8.71	3.44 ±0.13	2.33±0.03	75.40±7.23	137.80±7.39	24.76±0.62	0.32±0.02	237.80±4.39	2.16±0.29	2297.80±26.39
T4	80	163.20±8.91	3.32 ±0.25	2.26±0.10	74.20±5.60	139.60±7.38	27.52±0.52	0.33±0.04	239.60±6.38	2.00±0.92	2319.60±32.38
T5	90	169.00±9.26	3.09 ±0.50	2.05±0.07	69.40±7.97	142.60±8.35	28.66±0.99	0.29±0.05	242.30±5.35	2.02±0.22	2312.30±25.35
T6	100	166.00±6.15	3.49 ±0.06	2.44±0.07	78.40±3.44	145.20±4.14	26.86±0.57	0.29±0.03	245.20±7.14	1.96±0.44	2315.20±27.14

Mean of five observations
* Non significant

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