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Research Article

Effects of Dietary Vitamin D3 Over-Supplementation on Broiler Chickens' Health; Clinicopathological and Immunohistochemical Characteristics

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ABSTRACT

Introduction: Vitamin D_3 is used as a supplement in the feeds of livestock, pets, and human infants. However, the presence of excessive Vitamin D_3 has been shown to cause toxicity in humans and animals. This study investigated the clinicopathological aspects of Vitamin D_3 toxicity in broiler chickens.

Materials and methods: The median lethal dose (LD_{50}) of Vitamin D_3 was estimated by the up-and-down method. To determine long term (21 days) toxic effects of oral Vitamin D_3 supplementation, 90 (14-day-old) IBL-80 unsexed chicks were randomly divided into three groups as A (control, received basal diet), B (basal diet + Vitamin D_3 at 16.67 mg/kg body weight daily), and C (basal diet + Vitamin D_3 at 33.33 mg/kg body weight daily).

Results: The findings indicated that broiler chickens tolerated a single oral dose of Vitamin D_3 up to 550 mg/kg body weight (22,000,000 IU/kg) without mortality. The results of long-term (21 days) oral supplementation of divided doses of Vitamin D_3 in broiler chickens (groups B and C) showed progressive emaciation, elevated hemoglobin, hypercalcemia, hypophosphatemia, and increased alkaline phosphatase activity. At necropsy, pale liver and kidneys, congestion and hardening of lungs, mild congestion in the brain, and soft bones were observed in Vitamin D_3 treated chicks (groups B and C). Microscopically, degeneration and metastatic calcification in lung parenchyma and peribronchiolar epithelium, coagulative necrosis and calcification in kidneys, and calcification with fibroplasia in proventriculus were detected. Lungs and kidneys showed a significant difference in calcification score between groups B and C. Broiler chickens from Vitamin D_3 treated groups (B and C) showed strong immunohistochemical expression of Calbindin D28K in the intestine and kidneys but weak expression in the lungs.

Conclusion: This study demonstrates that broiler chicks can tolerate very high levels of a single oral dose of Vitamin D_3. Toxic effects of prolonged exposure to Vitamin D_3 are due to over-expression of Calbindin D28k in the intestine and kidneys, disturbing the calcium and phosphorous homeostasis and leading to metastatic calcification of vital organs. This study supports that prolonged over-supplementation of Vitamin D_3 causes toxic effects; hence, appropriate dietary Vitamin D3 supplementation limits should be set.

1. Introduction

Vitamin D_3 is a fat-soluble vitamin, essential for regulation of calcium and phosphorus homeostasis in the body[1,2]. When plasma calcium levels fall, Vitamin D_3 stimulates the release of calcium from bones, limits calcium excretion by kidneys, and regulates calcium absorption from the intestine[3]. Absorption and transport of calcium through the enterocytes depend on the presence of carrier protein calbindin D28K[4,5,6]. Vitamin D is not a single compound but consists of several different compounds, among which vitamin D_3

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(cholecalciferol) and D_2 (ergocalciferol) are the most important[3,7]. Poultry requires Vitamin D in the form of Vitamin D_3, which occurs naturally in fish liver oil or can be synthesized through the irradiation of animal sterol. While Vitamin D_2 from plant sources is active for rats and most mammals, it exhibits very low activity for poultry[8]. Vitamin D_3 is almost 10 times more toxic than Vitamin D_2 and causes generalized calcification of soft tissues[9,10].

Vitamin D_3 is used as a supplement in the feeds of commercial livestock, pets, and human infants. However, excessive Vitamin D_3 has been shown to cause toxicity in pets, swine, and human infants[10-12]. Currently, Vitamin D_3 is also used as a rodenticide (0.075% cholecalciferol), accidental ingestion of which is the most common source of Vitamin D_3 toxicity in dogs and cats[13].

Broiler diets are routinely supplemented with minerals and vitamins to overcome certain deficiencies[8]. Unfortunately, most of the vitamins are over-supplemented in commercial broiler diets to avoid losses due to processing and storage. In broiler nutrition, it is common practice to enhance feed formulations with Vitamin D_3 levels exceeding the recommended 200 IU (up to 4-10 times the minimum requirement as outlined by NRC[8]). This fortification aims to address prevalent bone issues, enhance bone quality, reduce footpad and hock dermatitis intensity, promote weight gain, optimize growth performance and immune response, as well as enhancing the tenderness, juiciness, and flavor of matured meat[14-18]. Several authors have reported the beneficial effects of dietary Vitamin D_3 at levels much higher than those recommended by the NRC in terms of performance indices, dressing percentage, breast meat yield, bone mineralization, and low incidence of tibial dyschondroplasia[19-24].

Considering the aforementioned studies, the utilization of basal levels of dietary Vitamin D_3 is infrequent, posing a potential hazard of over-supplementation[6,25]. Nonetheless, it has been observed that young broiler chicks exhibit remarkable tolerance to elevated Vitamin D_3 levels, such as 50,000 IU/kg[26]. Current observations have revealed that broilers can tolerate Vitamin D_3 concentrations as high as 100,000 IU/kg body weight, exhibiting no discernible signs of toxicity or mortality[6]. Persia et al.[27] reported that a diet supplemented by Vitamin D_3 at 2,200 to 102,200 IU/kg had no significant effect on the quality of egg shells or bones in laying hens. On the other hand, NRC indicated that Vitamin D_3 toxicity in poultry causes anorexia, reduced eggshell quality, egg production, egg weight, renal tubular calcification, muscular atrophy, and emaciation[8]. There is a knowledge gap about the oral LD_{50} (median lethal dose) and maximum tolerable dose of Vitamin D_3, including toxicological and clinicopathological effects of long-term Vitamin D_3 toxicity in poultry.

With this in mind, the current study was designed to determine oral LD_{50} of Vitamin D_3, tolerance of broilers for Vitamin D_3, and long-term toxic effects of excess Vitamin D_3 in broilers.

2. Materials and Methods

2.1. Ethical approval

All experiments were approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Punjab, India.

2.2. Broiler chick management

One-day-old broiler chicks (strain IBL-80, n = 120, 90+30) were obtained from the hatchery of the Department of Animal Breeding and Genetics (AGB), College of Veterinary Science, Guru Angad Dev Veterinary and Animal Science University (GADVASU), Ludhiana, Panjab, India. Broiler chicks were housed in cages, provided *ad libitum* drinking water, and standard broiler feed (NRC)[8] obtained from Godrej Agrovet Ltd, Khanna, Panjab, India (Table 1).

Table 1. Chemical composition of the control diet fed to broiler chicks

Constituent	Amount (%)	Constituent	Amount (%)
Vitamin D	4000 IU	Methionine + Cystine	1.0%
Crude Protein	21.5%	Linoleic acid	1.5%
Crude Fiber	4%	Arginine	1.45%
Crude Fat	4.5%	Calcium	1.2%
Lysine	1.2%	Available phosphorus	0.6%
Methionine	0.5%	Metabolizable Energy	3,100 kcal

%: values per 100 gm feed

2.3. Chemicals

Vitamin D_3 was purchased from Sigma Life Science, St. Louis, USA. A stock solution (100 mg/ml) of Vitamin D_3 was prepared by dissolving Vitamin D_3 in groundnut oil. Stock solutions were stored at 4°C and brought to room temperature before dosing. Calbindin D28k was also purchased from Sigma (Cat. No. ZRB1778).

2.4. Experimental Protocol

2.4.1. LD_{50} estimation and dose selection

An experiment was designed to estimate the acute (24 hours) oral LD_{50} of Vitamin D_3 in broilers using the up-and-down method[28]. Broiler chickens were acclimatized for 7 days. On day 8 onwards, five chickens were inoculated with a single oral dose each of Vitamin D_3, started from 9.5 mg (380,000 IU)/kg body weight, using the computer program AOT425 statpgm created by the United States Environmental Protection Agency (USEPA)[29] based on an iterative dose-selection algorithm. The next dose given to another five broiler chickens was recommended by the computer program based on the short-term (24 hours) lethality results (Table 2). The trials were carried out using the rule, increase the dose following a negative response [Survived (O)] and decrease the dose following a positive response [dead (X)]. Inoculated chickens were examined

Table 2. Protocol and observations of the LD_{50} estimation in broiler chickens

Dose/Kg BW* (mg/IU)	Age (Days)	Weight (Gram)	Total dose (mg/IU)	Observation		
				After 4 hours	After 24 hours	After 48 hours
9.5/380000	8	120	0.95/38000	No death	No death	No death
17.5/700000	9	120	2.1/84000	No death	No death	No death
55/2200000	10	120	6.6/264000	No death	No death	No death
175/7000000	11	200	38.5/1540000	No death	No death	No death
550/22000000	12	200	110/4400000	No death	No death	No death
550/22000000	13	250	137.5/5500000	No death	No death	No death

*BW: Body weight

Table 3. Experimental design showing treatment and description of broiler chicken supplemented with different levels of Vitamin D_3

Group	Treatment (14 DOA onwards)	21 DOA*	35 DOA*
A (n=3x10)	Control chicks were given only oil with feed and water *ad libitium*	15 chicks were sacrificed (5 chicks from each replicate)	15 chicks were sacrificed (5 chicks from each replicate)
B (n=3x10)	Chicks were treated with vitamin D_3 orally (in oil) at 16.67mg/kg BW daily	15 chicks were sacrificed (5 chicks from each replicate)	15 chicks were sacrificed (5 chicks from each replicate)
C (n=3x10)	Chicks were treated with vitamin D_3 orally (in oil) at 33.33mg/kg BW daily	15 chicks were sacrificed (5 chicks from each replicate)	15 chicks were sacrificed (5 chicks from each replicate)

* DOA: Days of age, BW: Body weight

daily for any overt clinical signs or mortality. Inoculated chickens displaying any signs of severe illness were euthanized according to the Institutional Animal Ethics Committee (IAEC) protocol and guidelines from Research Animal Resources (RAR) at GADVASU, Ludhiana, India. All the inoculated chickens used were humanely euthanized (after 48 hours of each dose) for necropsy and sample collection for histopathology.

2.4.2 Long-term effects of the study plan

After acclimatization for 2 weeks, 14 days old broiler chicks were tagged and randomly divided into 3 groups (Table 3). Group A (n = 30) served as the control group, Group B (n = 30) had 3 replicates of 10 broiler chickens, each treated with Vitamin D_3 orally (in oil) at the rate of 16.67mg/kg body weight (BW) daily, and Group C (n = 30) had 3 replicates of 10 broiler chickens each treated with Vitamin D_3 orally (in oil) at the rate of 33.33mg/kg BW daily. Broiler chickens were weighed daily before dosing to estimate the dose and volume of Vitamin D_3 dissolved in oil given to each chicken. Chickens were examined daily for any overt clinical signs or mortality. Broiler chickens displaying signs of severe illness were humanely euthanized according to the Institutional Animal Ethics Committee (IAEC) protocol and guidelines of GADVASU. Fifteen chicks from each group (5 chicks from each replicate) were euthanized at 21 (after 7 days of treatment) and 35 days of age (DOA, after 21 days of treatment). Body weights were recorded at euthanasia before blood and tissue collection. At necropsy, gross lesions were noted, followed by tissue collection from all visceral organs for histopathology and immunohistochemistry.

2.5. Hematobiochemical parameters

Blood samples (3-5 ml) from 15 experimental chickens in each group (at each euthanasia time point) were collected in heparinized vials. One aliquot was immediately processed for estimation of hemoglobin (Hb) by Cyan-methemoglobin method[30]. The other aliquot was centrifuged at 2000xg, 4°C for 15 minutes to obtain plasma and kept frozen for further analysis of various analytes, such as plasma calcium, plasma phosphorous, albumin, total protein, and alanine transaminase (ALT), aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) enzyme activity The analysis was performed using BIOTRAN BTR-830 auto analyzer, Autopak (Bayer Diagnostics India Limited, Gujarat, India) and Labkit (CHEMELEX, S.A. Spain) diagnostic reagent kits.

2.6. Histopathology and immunohistochemistry

At necropsy, tissue samples from visceral organs were collected in 10% neutral buffered formalin and processed for routine histopathology. Calcification in tissue sections was demonstrated by Von Kossa stain[31]. Paraffin-embedded tissues containing lungs, intestine, and kidneys were subjected to Immunohistochemistry (IHC) staining to demonstrate calbindin protein following an established method[6]. The antibodies used in IHC were anti-Calbindin D28k (primary antibody purchased from Sigma, USA) and a biotinylated secondary antibody[6].

2.7. Statistical analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA), and all data were analyzed by SPSS software (SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. Chicago, SPSS Inc.). The results were presented as mean ± SE (standard error). Calcification scores were statistically analyzed by the Mann-Whitney U test. Results were considered significant if $p < 0.05$.

3. Results and Discussion

3.1. LD_{50} and dose selection of Vitamin D_3

After acclimatization for 7 days, increasing doses of Vitamin D_3 dissolved in oil were given orally to 8-day-old broiler chickens [from 9.5mg/kg (380,000 IU/kg) to 550mg/kg (22,000,000 IU/kg)]. No adverse clinical signs

Table 4. Estimation of acute oral (24h) LD_{50} of Vitamin D_3 in broiler chicks

Variables	Result
LD50	>550 mg/kg BW orally
Initial dose	9.5
Last dose	550
Dose sequence	9.5, 17.5, 55, 175, 550, 550
Outcome	OOOOOO*

*(X = death; O = survival)

or mortality was observed within 24-48 hours of dosing (Table 4). All the chickens used in this experiment were euthanized for necropsy, and their tissue samples from all visceral organs were collected for histopathological examination. At necropsy, no gross pathological changes were observed. Microscopically, no pathological changes or calcification were observed in any visceral organ. These results indicated that broiler chicks in the current study could tolerate high levels of a single oral dose of Vitamin D3. The results suggest that a single oral dose of Vitamin D_3 can not cause toxicity in broilers. Hence, LD_{50} of Vitamin D_3 in broiler chicks is > 550mg/kg body weight. Vitamin D_3 is supplied in the feed throughout the growing phase of broilers. It was intriguing to see if small doses of Vitamin D_3 inoculated in chickens for 21 days can cause toxicity. Thus, the authors decided to inoculate a cumulative dose of Vitamin D_3 less than 550mg/kg BW and more than 550mg/kg BW divided into 21 doses inoculated daily in oil to find out the level and the time chickens start manifesting Vitamin D_3 toxicity. Therefore, dose rates of 16.67 mg/kg BW and 33.33 mg/kg BW were selected and inoculated per day for 21 days that turned out to be a total dose of 350 mg/kg BW and 700 mg/kg BW, respectively.

Vitamin D_3 at higher doses is toxic, but broilers have a tolerance for 50,000 IU/kg of Vitamin D_3 with no apparent negative effects on bone mineralization and growth[26] and no mortality up to 1,00,000 IU/Kg BW[5]. Renal tubular calcification is the most sensitive index of Vitamin D toxicity where 25-(OH)D_3 at 0.1mg/ kg of diet causes initial calcification[32]. No overt signs of toxicity or mortality were observed in poultry treated with Vitamin D_3 at 15000 IU/kg[33] or as high as 102,200 IU/kg of diet[27]. In the present study, a single oral dose as high as 550mg/kg BW (22,000,000 IU/kg BW) of Vitamin D_3 did not cause any calcification in vital organs or any apparent adverse clinical effects on broiler chickens. This difference could be due to a single oral dose, or some unknown inherent characteristics of broiler strain used in this study which is beyond the scope of this study. However, the study suggests that chicks can tolerate very high levels of single oral doses of Vitamin D_3 without any mortality or overt clinical signs.

3.2. Long-term effects

3.2.1. Clinical signs

Broiler chickens in all groups had normal feed and water intake and were alert at the start of the experiment. At 18 DOA, (day 5 of treatment), the treated chicks showed watery feces (Figure 1, more severe in Group C than Group B), were dull and recumbent, showed decrease in

Figure 1. The Vitamin D_3 intoxicated broiler chicks standing on the hocks, curling of the toe (arrow), and watery feces (arrow).

feed intake, polydipsia, and polyuria. These clinical signs became more severe during the treatment. At 28 DOA (day 14 of treatment) and onward, Group C stopped taking feed, and they were dull, reluctant to move with rigid limbs, resting on the hocks (Figure 1), ruffled body coat, severe progressive emaciation, dehydration, and weakness.

Kumar et al.[6], Schoemaker et al.[34], and Machlin[35] reported anorexia, polyuria, polydipsia, regurgitation, weight loss, depression, and renomegaly in broilers. In a hypercalcemic state, the ability of the renal tubules to respond to anti-diuretic hormone decreases, thus inhibiting the absorption of water, causing polyuria and polydipsia[36]. Metastatic calcification in the kidneys caused by hypercalcemia damages renal tubules, leading to decreased reabsorption of water[6]. Other clinical signs include muscle weakness, painful joints, demineralization of bone, and disorientation. These abnormalities eventually lead to death[37]. Morrow reported that an unregulated increase in plasma calcium and phosphorus levels in Vitamin D_3 toxicity causes mineralization in kidneys, GIT, cardiac muscles, skeletal muscles, blood vessels, and ligaments, causing structural damage leading to decreased functional capacity[38]. The loss of function contributes to developing ongoing end-stage clinical signs. In the present study, hypercalcemia, hypophosphatemia, and mineralization of various organs are more severe than in previously reported studies[6]. Thus, it elucidates that the clinical signs were the sequelae of severe biochemical and histopathological changes due to Vitamin D_3 toxicity.

Similar clinical signs, such as anorexia, diarrhea, progressive emaciation/weight loss, dehydration, weakness, and difficulty in movement were reported in different species by Roberson et al.[12], Long[10], Braun et al.[39], Radostits et al.[40] , and Sandhu and Brar[41].

3.2.2. Body weight

The mean body weight (MBW) of broiler chickens is summarized in Table 5. At 21 DOA (after 07 days of treatment), the MBW of chickens in Group C was significantly less than groups A and B ($p < 0.05$). The

Table 5. Effect of oral administration of vitamin D_3 on body weight of broiler chicken

Age (Days)	Body weight in grams (mean ± S.E)		
	Group A	Group B	Group C
14	282±11.43	283±11.16	278±12.58
21	410±15.81^b	388±13.56^b	313±16.55^a
35	778±43.97^c	432±26.53^b	300±27.01^a

Group A: Control feed, Group B: Control feed + vitamin D_3 at 16.67mg/kg body weight, Group C: Control feed + vitamin D_3 at 33.33mg/kg body weight, S.E: Standard error of mean; $^{a, b}$ means different superscripts within a row have significant differences at $p < 0.05$.

Group C chickens showed progressive emaciation and decrease in their body weight in comparison to Group B and Group A with course of treatment. The MBW of chickens in Group C was 31% less than Group A (control) and 23.96% less than chickens in Group B. At 35 DOA (after 21 days of treatment), the MBW of chickens in groups A, B, and C differ significantly, where the treated broiler chickens (groups B and C) showed decreased body weight gain ($p < 0.05$, Figure 2). Group C broiler chickens showed more severe progressive emaciation than Group B. The MBWs of chickens in Group C were 159.3% and 44% less than chickens in groups A and B, respectively.

Figure 2. Difference in the size and body weight of Vitamin D_3 intoxicated broiler chicken from Group C in comparison to the control group

The findings in the present study are in accordance with the findings of Kumar et al.[6] and Morrissey et al.[42] in broilers and Chavhan et al.[43] in rats. The harmful effect of high doses of Vitamin D_3 reduces growth and body weight gain.

Kumar et al.[6] reported the MBW of broiler chickens treated with Vitamin D_3 decreased by 51.69%, compared to MBW of broiler chickens of the control group. However, 29% BW loss was reported by Harrington and Page[44] due to Vitamin D_3 toxicity in horses. Increased plasma calcium and phosphorus in Vitamin D_3 toxicity cause mineralization of tissues which contributes to the development of clinical signs leading to inappetence and reduced BW gain[38].

3.2.3. Hematobiochemical parameters

The results of hematobiochemical parameters are summarized in Table 6. Treated broiler chickens in groups B and C showed hemoconcentration, hypercalcemia, and hypophosphatemia on days 21 and 35 ($p < 0.05$). The plasma total protein, albumin, ALT, and AST did not differ significantly in control (A) and treatment groups (B and C), but treated groups showed an increase in ALP activity where the plasma ALP activity of Group B was more than Group C at 21 and 35 DOA ($p < 0.05$).

Hemoconcentration in the present study agrees with Chavhan et al.[43] and Roberson et al.[12], who reported an increase in hematocrit in Vitamin D_3 toxicity in rats and lambs, respectively. Increased hematocrit contrasts with the results reported by Kumar et al.[45] where no difference was observed between the chickens of treatment and control groups. This may be due to a lower dose of Vitamin D_3 inoculated by Kumar et al.[45]. This increase in hemoglobin concentration may be due to dehydration caused by polyuria which could not be compensated by polydipsia.

Normal plasma/serum calcium and phosphorous concentration was 9-10.5 mg/dL and 5-7 mg/dL in chickens, respectively[46]. Taylor et al.[47] and Kumar et al.[6] reported that chickens given a toxic dose of Vitamin D_3 showed an increase in plasma calcium and a decrease in plasma inorganic phosphorus. Similarly, Vitamin D_3 toxicity studies in other species also reported hypercalcemia[6,40,43,48] and hypophosphatemia[49]. In the present study, hypercalcemia and hypophosphatemia in Vitamin D_3 treated chickens are primarily related to the role of Vitamin D_3, which regulates plasma calcium and phosphorous[50]. Vitamin D_3 increases calcium absorption from gut, excessive calcium mobilization from bone, and decreases calcium excretion from kidneys[51-53], leading to increased plasma calcium and

Table 6. Effects of oral administration of vitamin D_3 on hematobiochemical parameters in broiler chickens

Parameter	Group A (Mean ± S.D)		Group B (Mean ± S.D.)		Group C (Mean ± S.D.)	
	21 DOA*	35 DOA*	21 DOA*	35 DOA*	21 DOA*	35 DOA*
Hemoglobin (g/dl)	8.73±0.36^a	9.67±0.16^a	12.33±0.23^b	12.41±0.22^b	12.08±0.32^b	12.71±0.80^b
Calcium (mg/dL)	10.90±0.05^a	10.05±0.22^a	11.34±0.10^{ab}	15.03±0.06^b	11.64±0.19^b	15.20±0.33^b
Phosphorus (mg/dL)	5.26±0.04^b	5.90±0.04^b	3.99±0.02^a	4.66±0.11^{ab}	3.94±0.04^a	4.32±0.13^a
Albumin (g/dL)	2.61±0.05^a	2.71±0.06^b	2.41±0.08^a	2.42±0.16^b	2.52±0.15^a	2.62±0.17^b
Total protein (g/dL)	5.79±0.05^b	5.87±0.03^a	5.85±0.03^b	5.76±0.15^a	5.75±0.12^b	5.93±0.08^a
ALT (U/L)	8.02±0.11^a	8.48±0.16^a	7.54±0.18^a	8.51±0.19^a	7.54±0.18^a	7.74±0.39^a
AST (U/L)	196.19±.79^a	250.55±12.41^a	200.61±3.56^a	217.72±17.42^a	215.62±13.0^a	242.98±12.47^a
ALP (U/L)	499.20±6.60^a	509.00±3.63^a	771.20±60.32^b	1293.4±30.76^c	526.60±19.86^a	956.60±48.87^b

Group A: Control feed, Group B: Control feed + vitamin D_3 at 16.67mg/kg body weight, Group C: Control feed + vitamin D_3 at 33.33mg/kg body weight
ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: Alkaline phosphatase, * DOA: Days of Age
$^{a, b}$ values within a row with different superscripts differ significantly at $p < 0.05$.

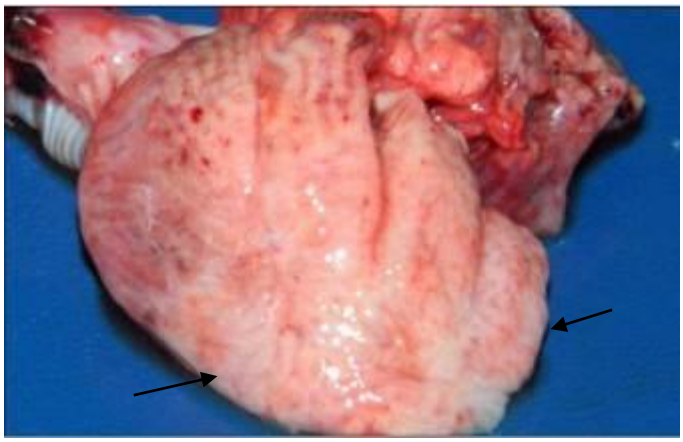

Figure 3. Gross lesions in Vitamin D_3 toxicity in lungs in broiler chickens from group C. Note the presence of white deposits of mineralization in lung (black arrows)

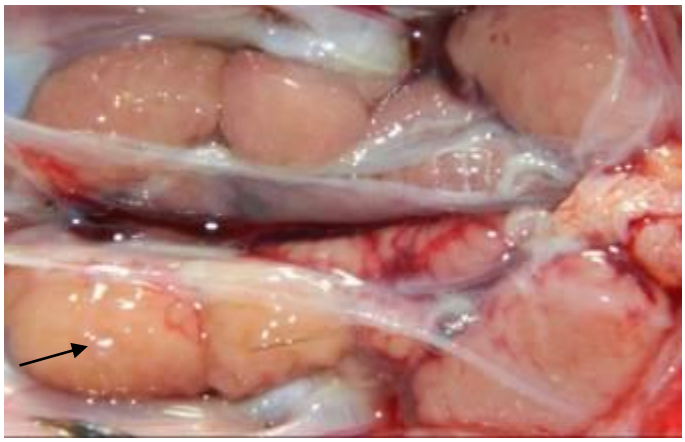

Figure 4. Gross lesions in Vitamin D_3 toxicity in the kidney in broiler chickens from group C. Note the yellowish-white discoloration of kidneys along with nephrosis (black arrows)

decreased plasma inorganic phosphorus which may cause death due to kidney and heart failure[40,48,54,55].

Normal total protein and albumin levels in the present study contrast with the findings of Roberson et al.[12] in lambs and Beasley[48] who reported proteinuria and low plasma total protein in Vitamin D_3 toxicity. This difference may be due to uricotelic excretion in chickens. Uricotelic excretion in chickens plays a crucial role in water conservation and prevents proteinuria. By excreting waste products in the form of uric acid instead of urea, chickens minimize water loss since uric acid requires less water for its excretion. This adaptation allows chickens to eliminate nitrogenous waste while conserving precious water resources efficiently. Additionally, uric acid has a lower risk of causing proteinuria, which is the presence of excessive protein in the urine. By avoiding proteinuria, chickens maintain proper kidney function and prevent potential damage to the renal system. Moreover, ALT and AST enzymes did not show any alteration due to the absence of pathological changes in the liver of the treated groups (B and C), as evidenced by histopathology.

The increase in the plasma ALP activity of Vitamin D_3-treated chickens is probably due to the rise in bone osteoblast cell activity. The osteoblast of bone is another target of Vitamin D_3 and plays a significant role in short-term calcium homeostasis. Vitamin D_3 is required for normal bone mineralization during skeletal growth and remodeling of bone. Vitamin D receptors in bone are located in osteoblasts and progenitor cells of bone and control the synthesis and secretion of alkaline phosphatase[37]. The elevated plasma ALP activity observed in Group B, compared to Group C, can be attributed to reduced osteoblast cell activity and an elevated level of osteoclast cell activity in the bones, likely due to the administration of a very high dose of Vitamin D_3 in Group C. Very high doses of Vitamin D_3 (>100 times the normal dose) can lead to a negative calcium balance, causing accelerated bone resorption, similar to the toxic effects observed in cases of *Solanum malacoxylon*, *Cestrum diurnum*, and *Trisetum flavescens* toxicity[37].

3.2.4 Gross post-mortem changes

Group A (control) chickens did not show any gross lesions at necropsy. Vitamin D_3-treated chickens showed decreased body weight, emaciation, and ruffled body coat. Group C chickens were more severely affected than Group B. They showed more prominent lesions of Vitamin D_3 toxicity like pale liver, congested lungs and felt hard and gritty when cut during necropsy (Figure 3), pale kidneys (Figure 4), congestion in the brain, and soft bones. Other organs did not reveal any significant gross lesions.

Kumar et al.[6] reported similar gross observations like a decrease in BW, emaciation, ruffled body coat, soft bones, focal hemorrhages, lung congestion, pale white kidneys with focal hemorrhages, and mild congestion of brain and pancreas in chickens treated with Vitamin D_3.

3.2.5. Histopathological findings

Group A (control) chickens did not show any microscopic lesions of Vitamin D_3 toxicity or calcification. Histopathological changes in the organs of chickens affected with Vitamin D_3 toxicity were more pronounced in Group C than in Group B and were more severe in chickens at 35 DOA. Calcification scores (summarized in Table 7) were higher in broiler chickens from Group C than Group B, where calcification was more evident in Group C than Group B. Diffuse calcification of affected organs was observed in Vitamin D_3 treated broiler chickens at 35 DOA (Group C > Group B).

3.2.5.1. Lungs

At 21 DOA, there was mild lung parenchyma congestion with degeneration and bronchiolar epithelium necrosis leading to bronchioles denudation in groups B and C. Moreover, broiler chickens in group C showed emphysematous lungs.

Table 7. Comparative calcification of different organs of broiler chicken supplemented with a toxic dose of Vitamin D_3

Organs	Groups				p-value	
	21 DOA		35 DOA			
	Group B	Group C	Group B	Group C	21 DOA	35 DOA
Lungs	+++	++++	++++	+++++	0.21	0.05
Bronchi	++	+++	+	++	0.03	0.03
Kidney	++	+++	+++	++++	0.01	0.01
Ureter	--	+	--	++	0.13	0.03
Proventriculus	+	++	+	++	0.10	0.02

Group B: Control feed + vitamin D_3 at 16.67mg/kg body weight, Group C: Control feed + vitamin D_3 at 33.33mg/kg body weight
Mean calcification score, -- = no change, + = slight, ++ = mild, +++ = moderate, ++++ = severe, +++++ = very severe

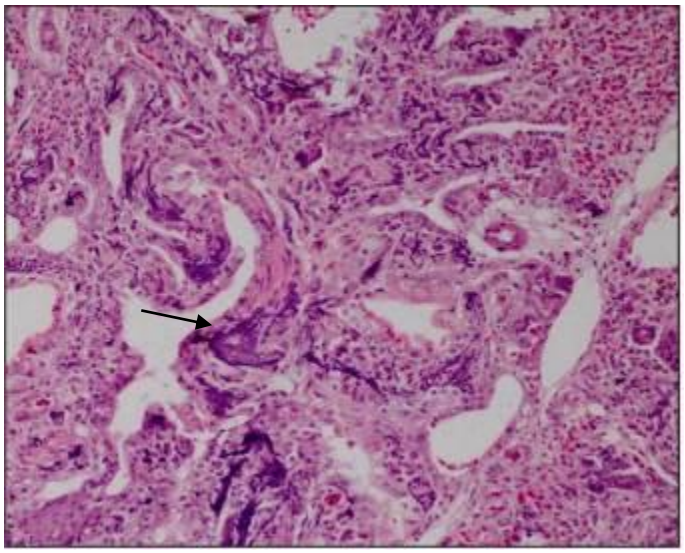

Figure 5. Section of lungs from group B showing alveolar septal thickening with marked diffuse calcification in the lung parenchyma (arrow). The lung parenchyma shows focal areas of hemorrhages. H&E X20.

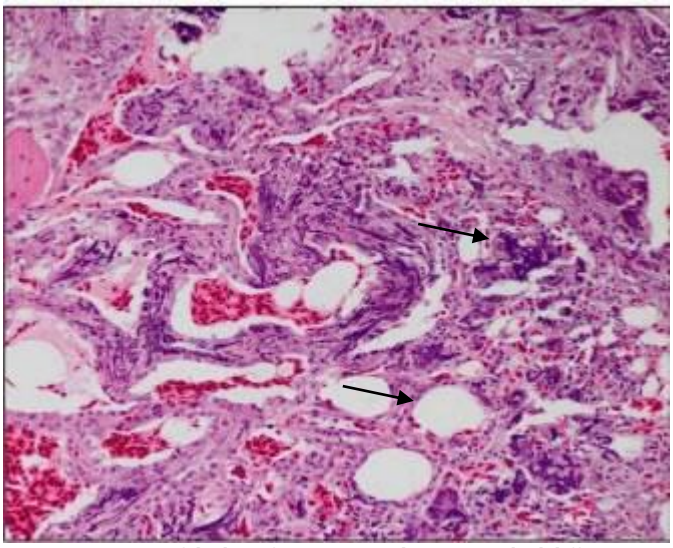

Figure 6. Section of the lung from group C showing marked diffuse thickening of interalveolar septae due to ensuing calcification (arrow) in the area and resultant atelectatic patches (arrow) in the areas. H&E X20.

There was moderate peribronchiolar calcification and fibrosis leading to the thickened alveolar septa in Group B whereas, marked and diffuse calcification in the alveolar wall leading to widespread areas of alveolar septal thickening and focal areas of fibrosis was observed in Group C. At 35 DOA, there were marked areas of alveolar congestion and hemorrhages with emphysema. Severe calcification of tunica muscularis of the vessel wall, along with mineral deposits in lung parenchyma were observed in Group B (Figure 5). In Group C, diffused areas of marked calcification of the alveolar wall were observed, and thickened alveolar septa were more pronounced than in Group B (Figure 6). Mineralization and calcification in the lungs were confirmed by the Von Kossa stain (Figure 7).

3.2.5.2. Bronchi

At 21 DOA, bronchiolar epithelium was slightly hemorrhagic and sloughed off with slight peribronchiolar calcification in Group B. In Group C, goblet cells of bronchiolar epithelium showed mild hyperplasia and hemorrhages with moderate to mild calcification of peribronchiolar epithelial cells. At 35 DOA, the section from Group B showed hemorrhages with slight peribronchiolar calcification. In Group C, bronchiolar epithelium indicated hemorrhages and hyperplasia of goblet cells with mild calcification in submucosa.

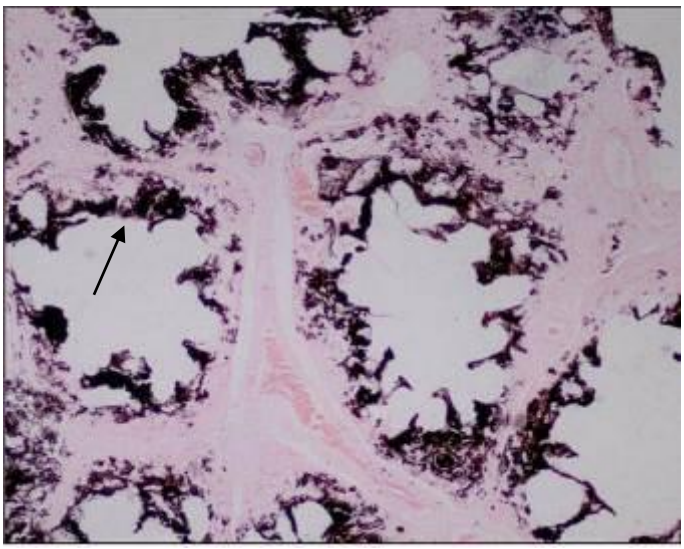

Figure 7. Section of the lung from group C showing black deposits of calcium within para bronchiolar epithelium (arrow). **Von Kossa X4.**

3.2.5.3. Kidney

At 21 DOA, Group B showed coagulative necrosis of renal tubular epithelium besides hemorrhages. Mild and patchy areas of metastatic calcification encircled by fibroplasia were observed in renal tubules and glomeruli. In Group C, renal tubular epithelium was degenerated and

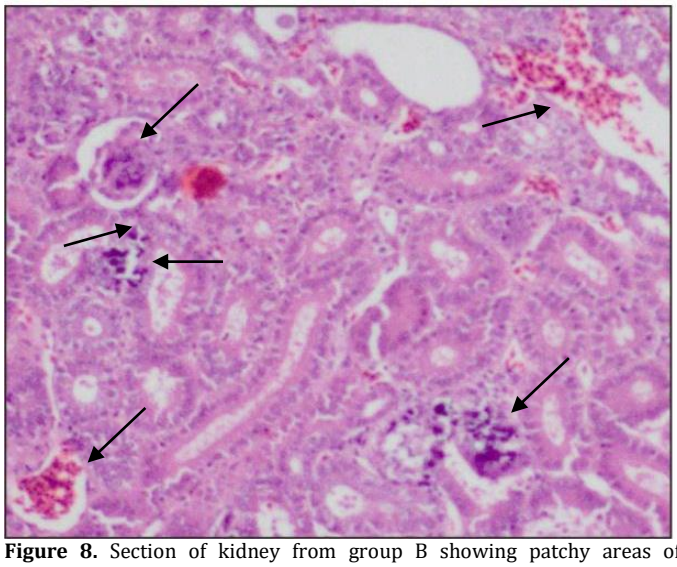

Figure 8. Section of kidney from group B showing patchy areas of calcification (arrow) along with areas of congestion and hemorrhage (arrow). H and E X20

necrosed. Multifocal areas of calcification in glomeruli and renal tubules were observed besides areas of marked congestion. Moreover, glomeruli increased cellularity and fibrotic changes. Focal areas of metastatic calcification were observed in collecting tubules, interstitium, and glomeruli. At 35 DOA, renal tubular epithelium showed focal areas of coagulative necrosis and hemorrhages in Group B. Renal parenchyma and glomeruli had moderate metastatic calcification (Figure 8). Group C showed vacuolar degeneration and necrosis of renal tubular epithelium and increased cellularity and fibrotic changes in glomeruli. Glomeruli and tubular epithelium showed severe metastatic calcification (Figure 9). Calcification in kidneys was confirmed by Von Kossa stain (Figure 10).

3.2.5.4. Ureter

At 21 DOA, no calcification was observed in the wall of ureter in Group B, whereas focal areas of slight calcification were observed in the ureter wall in Group C. At 35 DOA, no to slight calcification of the ureter wall was observed in Group B but in Group C, the ureter wall was mildly calcified.

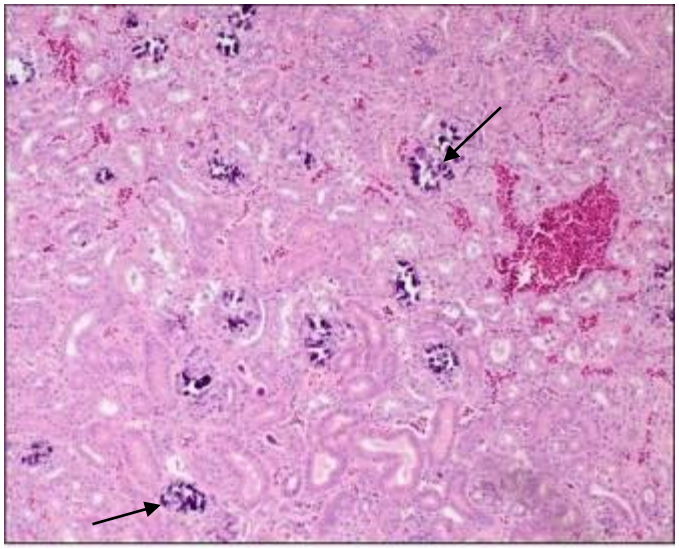

Figure 9. Section of kidney from Group C showing multifocal areas of calcification in the glomeruli as well as in tubules (arrow) besides areas of marked congestion. H&E X10.

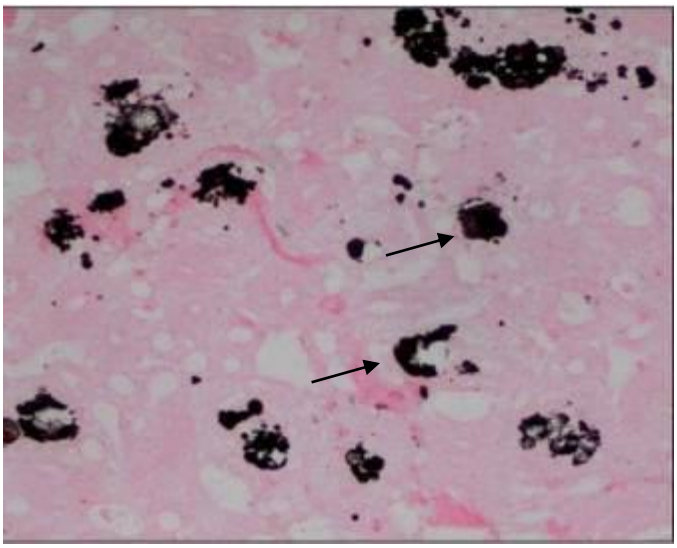

Figure 10. Section of kidney from group C showing black deposits of calcium within glomeruli and renal tubules (arrow). Von Kossa X4

3.2.5.5. Proventriculus

At 21 DOA, focal areas of slight calcification were observed in proventricular glands and tunica propria in Group B. In contrast, in Group C, tunica propria of muscularis mucosae showed marked diffuse calcification with fibrosis. At 35 DOA, vessel walls, tunica propria, and proventricular glands of chicks from Group B showed mild calcification (Figure 11), whereas in Group C, widespread areas of diffuse calcification were observed in proventricular glands with fibrosis in tunica propria (Figure 12). Calcification in proventriculus was confirmed by Von Kossa staining (Figure 13).

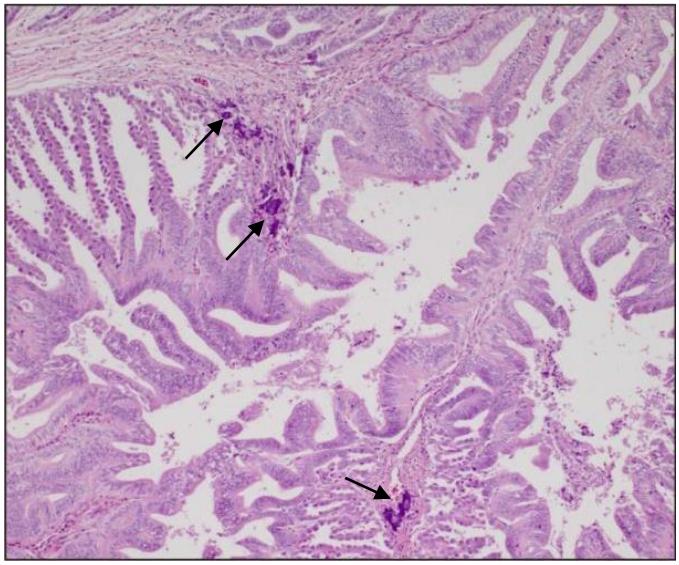

Figure 11. Section of proventriculus from Group B showing focal areas of calcification (arrow) in sub glandular connective tissue. H&E X20.

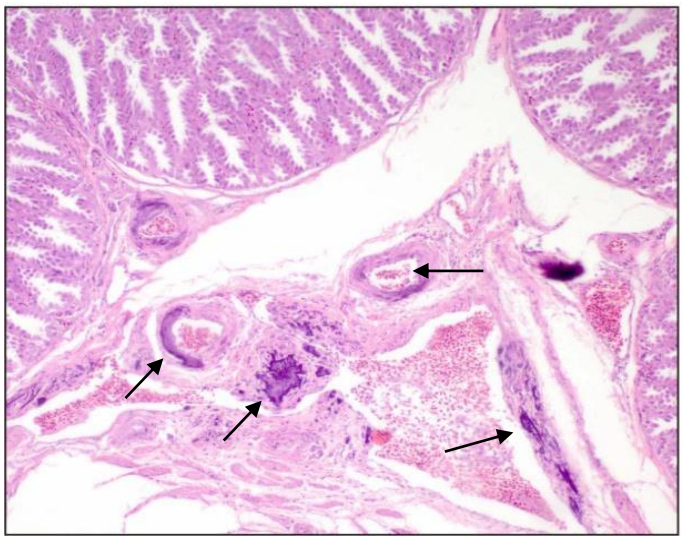

Figure 12. Section of proventriculus from Group C showing calcification in vessel wall (arrow) and sub glandular connective tissue (arrow). H&E X20.

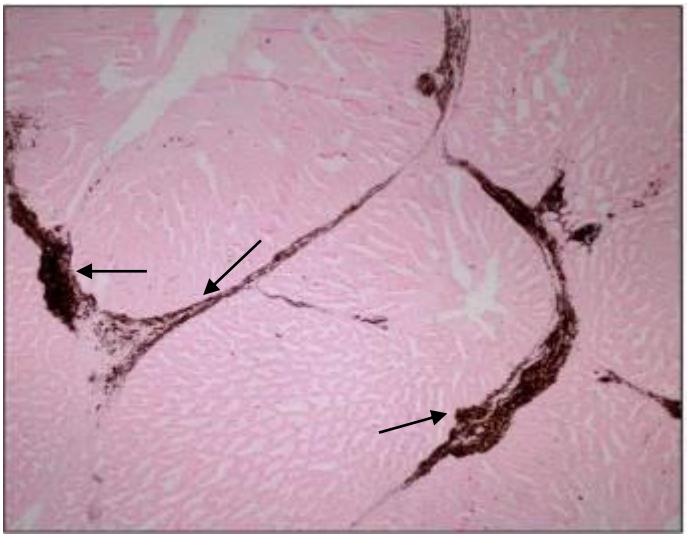

Figure 13. Section of proventriculus from Group C showing black deposits of calcium in sub glandular connective tissue (arrow). Von Kossa X4.

Other visceral organs did not show evidence of calcification or other histopathological changes of Vitamin D_3 toxicity.

3.2.5.6. Calcification scores

No calcification from Group A (control) broiler chickens was observed in the tissue section. At 21 DOA, the kidney and bronchi of chicks from Group C showed significantly higher calcification scores than Group B. At 35 DOA, lungs, bronchi, kidneys, ureters, and proventriculus showed significantly higher calcification in Group C than in Group B (Table 7).

Previous studies reported similar lesions in the lungs, bronchi, and kidneys in different species[9-11]. Hunt et al.[9] studied the toxicity of cholecalciferol in Rhesus monkeys (*Macaca mulatta*). They reported the presence of brown dirty color deposits of calcification within alveolar septum leading to septal thickening with infiltration of macrophages, lymphocytes, a few multinucleated giant cells, and neutrophils. Hunt et al.[9] reported mineralization of cortical tubular epithelium, their basement membrane, interstitium, in walls of renal blood vessels and within the epithelium of collecting ducts of kidneys. Morrissey et al.[42] reported renal tubular calcification when chicks were fed Vitamin D_3 at the rate of 10.0 mg/kg of diet and when 25-OH Vitamin D was fed at the rate of 0.1 mg/kg diet. Large white male turkey poults were fed a diet containing a high Vitamin D level (900,000 ICU/kg), causing hypervitaminosis D as evidenced by mild growth depression and renal tubular mineralization[56]. Kumar et al.[6] reported slight to mild calcification in kidneys and ureters of chicks treated with Vitamin D_3 at the rate of 2.5 mg/kg BW. Kumar et al.[6] observed similar histopathological changes in proventriculus as observed in this study, like calcification in proventricular glands, tunica propria, and connective tissue.

Beasley[48] and Price et al.[55] reported that 1,25-dihydroxy cholecalciferol, the active metabolite of cholecalciferol, causes hypercalcemia by increased resorption/ mobilization of calcium from bone, increased absorption of calcium from the intestine and decreased calcium excretion by the kidney. The effect of hypercalcemia on cells includes altered cell membrane permeability, altered calcium pump activity, decreased cellular energy production, and cellular necrosis. The net result is the deposition of calcium in unusual sites, metastatic calcification of vital organs causing structural damage that leads to their decreased functional capacity. As the toxicity increases, clinical signs include hypertension, polyuria, and extremely elevated serum calcium level may cause cardiac arrhythmias and death due to renal and cardiac failure[38].

3.2.6. Immunohistochemical expression of Calbindin D28k

The expression of calcium-binding protein (CaBP) calbindin D28k was studied in intestine, kidneys, and lungs. Strong immunohistochemical expression of calbindin D28k was observed in intestine (Figure 14) and kidney (Figure 15)

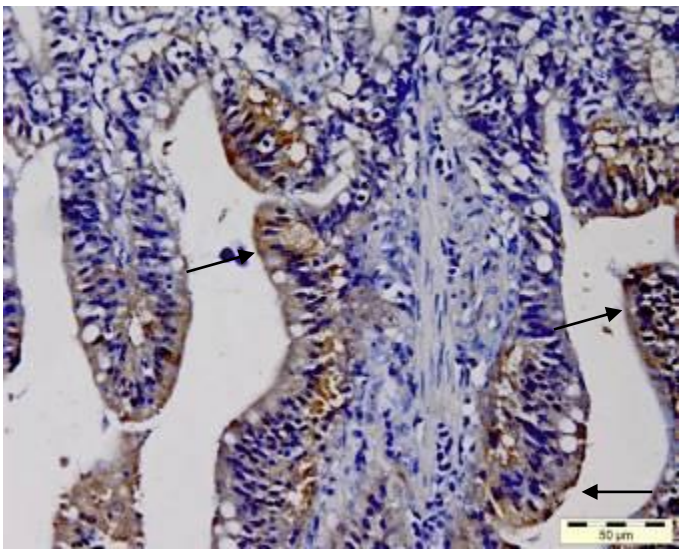

Figure 14. Section of intestine from Group C showing strong expression of Calbindin D28k in intestinal villi (brown color, arrow). IHC. X40.

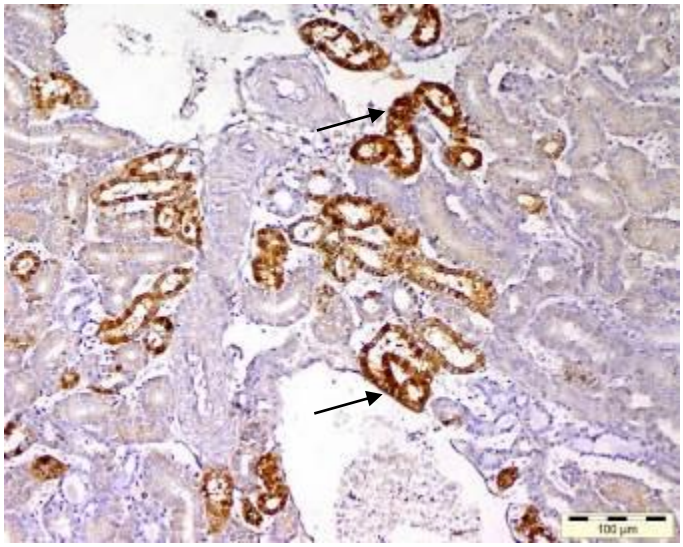

Figure 15. Section of Kidney from Group C showing strong expression of Calbindin D28k (brown color, arrow). IHC X20.

but weak expression was observed in lungs (Figure 16). The expression was stronger in broiler chickens from Group C than in Group B. Similar results have been observed for the immunocytochemical localization of calcium binding protein (CaBP) by Kumar et al.[6] and Roth et al.[57] in chickens treated with Vitamin D_3.

Calbindin immunoreactive sites were found to play a role in regulating intracellular calcium. The localization of calbindin-D28k in the intestine indicates that calbindin D28k is associated with calcium absorption from the intestine, and a higher dose of Vitamin D_3 induces higher calbindin-D28k expression.

In kidneys, calbindin-D28k plays a role in selective calcium reabsorption and regulating several cellular processes. These results indicate that calbindin D28k protein has a fundamental and specific role in maintaining cellular calcium homeostasis. The active transport of Ca through the enterocytes depends on the presence of the carrier protein calbindin D28K, which is involved in the intracellular transfer of Ca from the apical to the basolateral membrane[5,58]. Many studies have demonstrated a correlation between the intestinal expression of calbindin D28K and active, transcellular Ca absorption[59-61]. Vitamin D_3 metabolite $1,25(OH)_2D_3$ is most effective in regulating calcium homeostasis-associated gene expression[62]. The expression of calbindin in the intestine and kidney has been shown to be regulated by $1,25(OH)_2D_3$ in rats and chickens[63-65]. Supplementation of $1,25(OH)_2D_3$ could induce calbindin expression in the duodenum of mice, as reported by Hoenderop et al.[66].

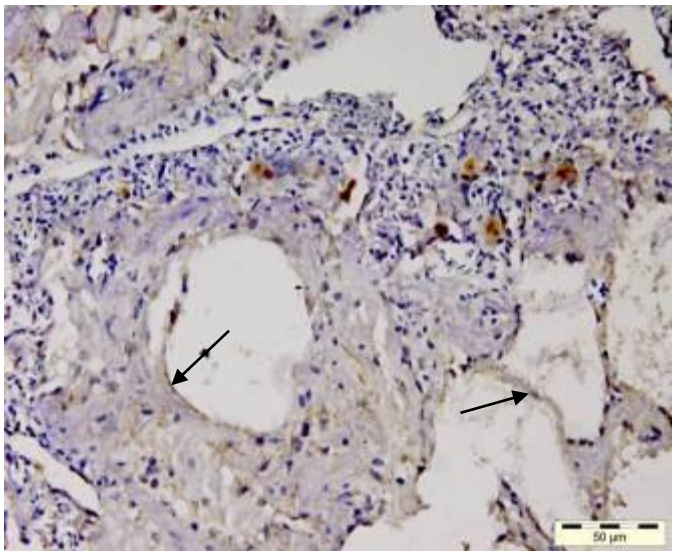

Figure 16. Section of lung from chicks of Group B showing weak expression of Calbindin D28k (brown color, arrow). IHC X20.

4. Conclusion

In the current study, the broilers were highly tolerant to single oral dose of Vitamin D_3 to the level of 550 mg (2,20,00000 IU) per kg BW but small doses fed over a longer period of time induces toxic effects. Vitamin D_3 shows its hormonal action by stimulating and activating Calbindin D28k protein. Calbindin D28K is responsible for calcium absorption and maintaining the Calcium homeostasis and Ca\P ratio in broilers. In cases of Vitamin D3 toxicity in broilers, there is a high expression of Calbindin D28k in the intestine and kidneys. This overexpression of Calbindin D28k results in excessive calcium absorption from these sites, leading to hypercalcemia and hypophosphatemia. Additionally, it causes alterations in the activity of ALP, which contributes to generalized metastatic calcification in various organs, with a particular predilection for the kidneys, lungs, and proventriculus. Along with these effects, Vitamin D3 toxicity can also induce other toxic effects in broilers. The authors suggest a cautious approach in supplementing broiler feed with Vitamin D_3 to prevent bone and leg issues or enhance meat quality. Instead, it can lead to toxic and adverse effects.

Declarations

Competing interest

The authors declare that they have no conflict of interest.

Authors' Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Rahul Kumar. The first draft was written by Rahul Kumar and Rajinder Singh Brar and Harmanjit Singh Banga commented on all versions of manuscript. All authors read and approved the final manuscript.

Funding

Not applicable.

Availability of data and materials

Data from the study are available according to a

reasonable request.

Ethical considerations

All experiments were approved by the Institutional Animal Ethics Committee (IAEC) of GADVASU, Ludhiana, India.

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