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Comparison between the protective effects of vitamin K and vitamin A on the modulation of hypervitaminosis D3 short-term toxicity in adult albino rats

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Background/aim: Vitamin D_3 has increased risk of toxicity due to its common use in multivitamin preparations. Vitamin K and vitamin A play an important role in vitamin D action. The goal of the current study was to compare the protective effects of vitamin K and vitamin A on the modulation of hypervitaminosis D_3 toxicity in rats by assessing serum calcium, renal function tests, cardiac enzymes, and related histopathological changes.

Materials and methods: Eighty adult albino rats were divided into four groups; each group consisted of 20 rats. The first group received water; the second received a toxic dose of vitamin D_3; the third received a toxic dose of vitamin D_3 with vitamin A; and the fourth received a toxic dose of vitamin D_3 with vitamin K.

Results: Vitamin D_3 toxicity led to significant abnormalities of cardiac enzymes, renal function tests, and serum calcium associated with histopathological changes in the kidney, heart, lung, adrenal gland, and aorta. Individual administration of vitamin A or vitamin K with a toxic dose of vitamin D improved the biochemical and histopathological abnormalities of hypervitaminosis D_3.

Conclusion: Vitamins A and K showed the same protective effects in the modulation of hypervitaminosis D_3 short-term toxicity.

Key words: Vitamin D_3, vitamin K, vitamin A, hypervitaminosis

1. Introduction

Vitamins are essential organic compounds for the normal growth and activity of the body. They are needed in small amounts. Most vitamins are not synthesized by the body, but are naturally found in foods that are obtained from plants and animals. Vitamins are either water-soluble or fat-soluble. Water-soluble vitamins are excreted more rapidly than fat-soluble vitamins (1).

Vitamin D is not a single compound, but two different fat-soluble compounds that have a vitamin D activity. Vitamin D_3 (cholecalciferol) is of animal origin and has the ability to prevent and cure rickets. Vitamin D_2 (ergocalciferol) is of plant origin. Vitamin D_3 is approximately ten times more toxic than vitamin D_2. The toxicity of vitamin D_3 causes generalized calcification of soft tissues (2).

Vitamin D is stored in the human body as calcidiol (25-hydroxy-vitamin D). It has a large volume of distribution and a half-life of about 20 to 29 days. Vitamin D and its metabolites maintain normal blood calcium levels by increasing the intestinal absorption of calcium, stimulating bone resorption, and decreasing the renal excretion of calcium. There are many foods rich in vitamin D, such as cod liver oil, salmon, mackerel, sardines, milk (full cream, fat-free, and reduced fat), liver, beef, and egg yolk (3).

Currently there is an increased risk of vitamin D_3 (cholecalciferol) toxicity because of its use in infant feed supplements and multivitamin preparations. Various plant species are a source of vitamin D_3 toxicity in livestock because of their high concentrations of vitamin D analogue. Vitamin D is the most toxic of all the vitamins; therefore, it is used in several countries for control of commensal rodents. All cases of vitamin D toxicity have involved the intake of over 1000 µg/day (40,000 IU). In the United States, there were 284 fatal cases of vitamin

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D overdose in 2004. The reported causes of death due to vitamin D_3 toxicity were cardiac and renal failure (4).

High doses of vitamin D_2 and vitamin D_3 lead to marked hypercalcemia and calcium salt deposition in soft tissues such as kidneys, blood vessels, heart, and lungs. Thus, soft tissues tend to become calcified while bone tends to be rarified. The mineralization of vital organs causes structural damage and decreases the functional capacity of these tissues and organs (5).

Vitamin K is a group of fat-soluble compounds. It is present as K_1 and K_2 in multivitamin food supplements and as K_3 and K_4 in drugs. It carboxylates a number of protein factors involved in the coagulation process, such as prothrombin, leading to the formation of calcium-binding sites on glutamyl side chains of proteins (gamma-carboxyglutamic acid (Gla)). Gla proteins (osteocalcins) are found in the bone and are involved in the mobilization and deposition of calcium. Matrix Gla proteins are found in other tissues such as the lung, kidney, and spleen (6).

Vitamin A is a fat-soluble compound and its requirements are expressed in retinol equivalent terms. It is considered an essential factor for vitamin D action. Vitamin D provided through the diet or by ultraviolet light depletes the blood levels and liver stores of vitamin A. Some studies indicated that the administration of large amounts of vitamin D causes relative avitaminosis A. Other researchers reported that vitamin A is required for the full functioning of vitamin D receptors. The combined effect of vitamins A, K, and D would be expected to protect against soft tissue calcification when vitamin D is taken in high doses (7).

The present study aimed to compare the protective effects of vitamin K and vitamin A in the modulation of hypervitaminosis D_3 short-term toxicity by assessing serum calcium, renal function tests, cardiac enzymes, and histopathological changes in the kidney, heart, lung, aorta, and adrenal glands.

2. Material and methods

Eighty healthy adult albino rats weighing 200–300 g were obtained from the animal house of King Abdel Aziz University, Jeddah, Saudi Arabia. The rats were exposed to a 12 h light/dark cycle. They had free access to water and food during the experimental period. The animals were divided into four groups; each group consisted of 20 rats. The first group (control) received distilled water; the second received an intraperitoneal single toxic dose of vitamin D_3 (2 mg/kg) (5); the third received an intraperitoneal single toxic dose of vitamin D_3 (2 mg/kg) with a single oral therapeutic dose of retinyl palmitate vitamin A (1000–9000 IU/kg daily) (8,9); the fourth received an intraperitoneal single toxic dose of vitamin D_3 (2 mg/kg) with a single oral therapeutic dose of vitamin K_1 (15 mg/kg per day) (10). The distilled water and all vitamins (D, A, and K) were administered for 1 month. The administration of distilled water and vitamins (A and K) was carried out by orogastric tubes. Vitamin D_3 (cholecalciferol) was available in ampoule form; each ampoule contained 5 mg in 2 mL, which is equivalent to 200,000 IU, produced by Memphis Pharmaceutical and Chemical Industries (Egypt). Vitamin K_1 (phytomenadione) is available in a 10 mg/mL solution in a 1 mL ampoule (Konakion), produced by Roche products (UK). Vitamin A is available in a 50,000 IU capsule form, produced by Kahira Pharmaceutical & Chemical Industries (Egypt).

2.1. Blood sample collection

At the end of the experiment, the rats were anesthetized with diethyl ether. Blood samples were obtained from the orbital sinus using capillary tubes. The samples were centrifuged at 3000 rounds at 4 °C for 10 min to separate the serum for assessment of renal biomarkers (urea and creatinine), serum calcium level, and cardiac enzymes (creatine kinase-MB (CK-MB), troponin I (cTnI), and myoglobin). Urea and creatinine were determined by routine colorimetric methods using commercial kits and quantified on a clinical biochemistry autoanalyzer (11). Serum calcium assay was performed by a calcium colorimetric assay kit at a concentration of 0.4–100 mg/dL (0.1–25 mM) and an absorbance of 575 nm (12). Measurements of cTnI and CK-MB mass were done using an Elecsys analyzer by the cTnI STAT third generation and CK-MB STAT methods (Roche Diagnostics, Germany). These assays are based on electrochemiluminescence immunoassay technology (ECLIA) using two mouse monoclonal antibodies in a sandwich-format, two-step assay. They were done on Elecsys 1010 and 2010 immunoassay analyzers according to the manufacturer's instructions (Roche Diagnostics). Myoglobin concentrations were determined using the respective Stratus fluorometric enzyme immunoassay (Dade Behring, USA) (13).

2.2. Histopathological studies

All the animals were sacrificed under excess anesthesia 24 h after the last administration of vitamins. An incision was made in the chest and abdomen for heart, lung, aorta, kidney, and adrenal gland excisions. Tissue specimens (heart, lung, aorta, kidney, and adrenal glands) were collected from the four groups and then fixed in 10% neutral-buffered formalin. The fixed specimens were trimmed, washed, and dehydrated in ascending grades of alcohol; cleared in xylene; embedded in paraffin; sectioned at 4 to 6-µm-thickness; and stained by hematoxylin and eosin and Von Kossa stain (14).

2.3. Statistical analysis

Statistical analysis was performed using SPSS version 17. The variability of the results was expressed as means ± standard deviation (SD). The results were analyzed using one-way ANOVA, a post hoc multiple comparisons test (Tukey's test), and a paired sample t-test to investigate differences among groups. A P value of 0.05 was considered significant.

2.4. Ethical considerations

The most appropriate animal species was chosen for this research. A high standard of care and animal well-being was promoted at all times. The appropriate sample size was calculated by using the fewest number of animals to obtain statistically valid results. Painful procedures were performed under anesthesia to avoid distress and pain. Our standards of animal care and administration met those required by applicable international laws and regulations.

3. Results

3.1. Histopathological observations

Photomicrographs are provided in Figures 1–10.

3.1.1. Pulmonary histopathological findings by a light microscope

Examination of lung sections from the control group (group 1) showed normal alveoli with thin interalveolar septa and clearly seen alveolar sacs (Figures 1a and 2a). However, the lung tissues of the second group of rats, which received 2 mg/kg of vitamin D_3, revealed significant changes in relation to the control group. These changes were in the form of calcium deposition in the alveolar and interalveolar septa, infiltration of lymphocytes, macrophages, occasional neutrophils, and a few giant cells in the thickened alveolar septa, marked hemorrhage, emphysema, and edema. Von Kossa staining of lung tissues showed calcified bronchi and other pulmonary tissues with

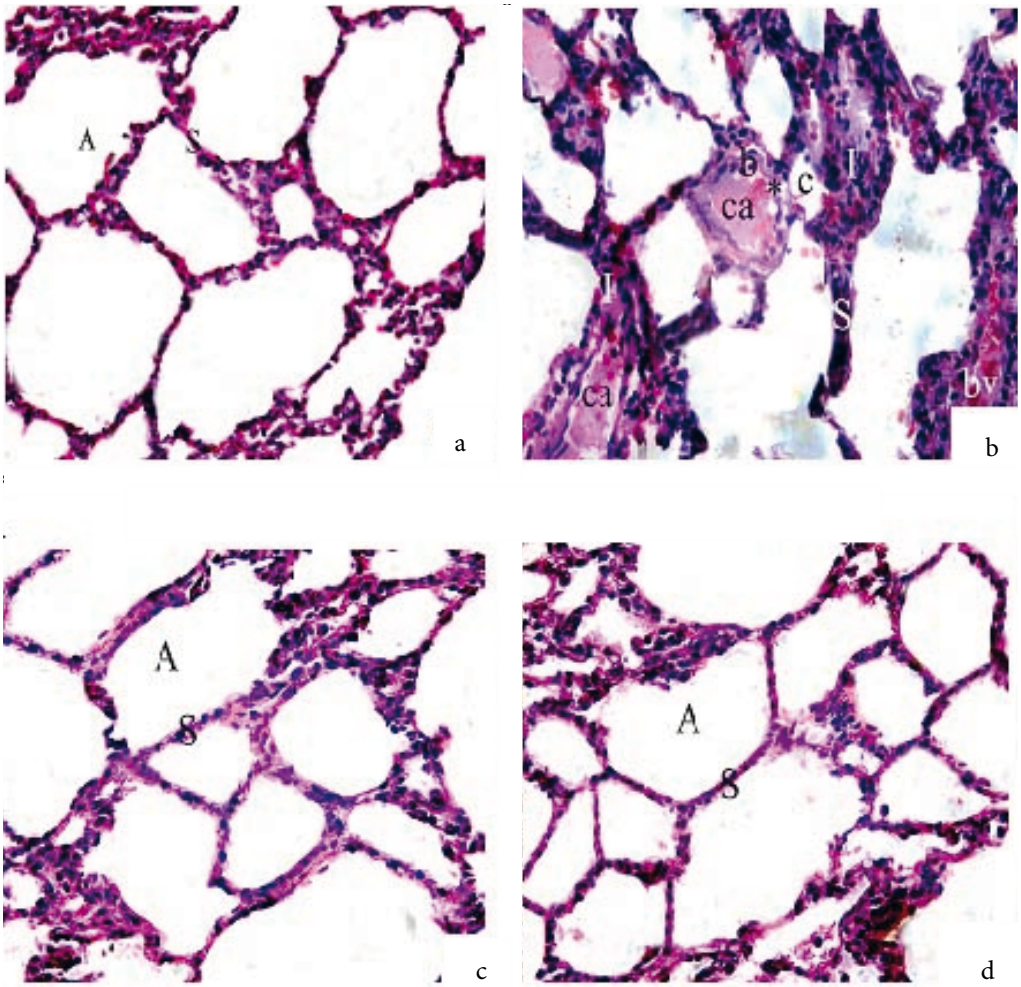

Figure 1. a) A photomicrograph of a transverse section in a control rat lung shows normal architecture of the alveoli (A) with thin interalveolar septa (S) (H&E, 400×); b) a photomicrograph of a transverse section in a rat lung from the second group shows loss of normal architecture, fragmentation, and degeneration of the alveoli with marked collapse of some alveoli (c); subsequent compensatory dilatation of other alveoli, marked thickening of the interalveolar septum (S) with numerous areas of cellular infiltration (I) in the connective tissue surrounding lung bronchioles (b); and thickened walls of the pulmonary blood vessels that contain hemorrhagic blood cells (bv) with deposition of calcium (ca) in the interalveolar septum and bronchial wall (black star) (H&E, 400×); c) a photomicrograph of a transverse section in a rat lung from the third group shows normal architecture of the alveoli (A) with thin interalveolar septa (S) (H&E, 400×); d) a photomicrograph of a transverse section in a rat lung from the fourth group shows normal architecture of the alveoli (A) with thin interalveolar septa (S) (H&E, 400×).

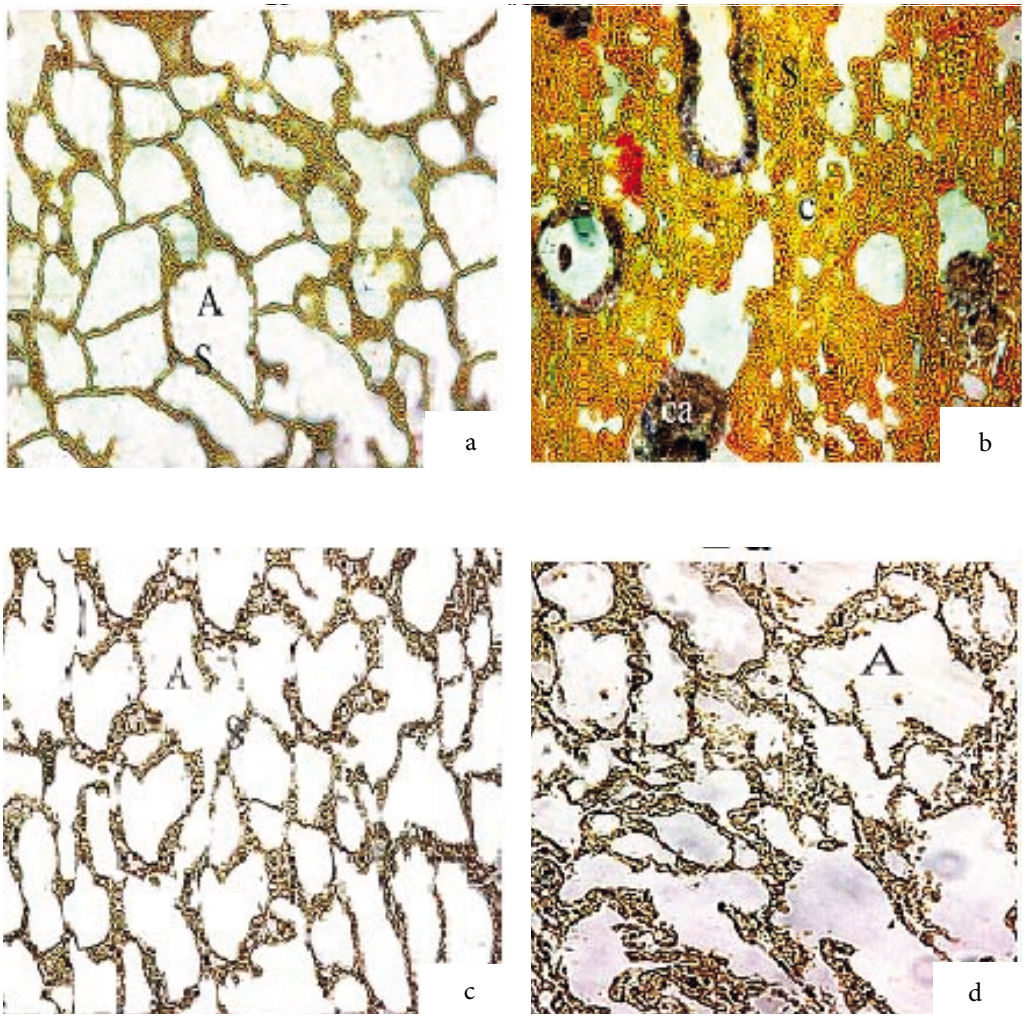

Figure 2. a) A photomicrograph of a transverse section in a control rat lung shows normal architecture of the alveoli (A) with thin interalveolar septa (S) (Von Kossa, 400×); b) a photomicrograph of a transverse section in a rat lung from the second group shows loss of normal pulmonary architecture, fragmentation and degeneration of the alveoli with marked collapse of some alveoli (c), and calcium deposition (ca) in the interalveolar septum and bronchial wall (Von Kossa, 400×); c) a photomicrograph of a transverse section in a rat lung from the third group shows normal architecture of the alveoli (A) with thin interalveolar septa (S) (Von Kossa, 400×); d) a photomicrograph of a transverse section in a rat lung from the fourth group shows normal architecture of the alveoli (A) with thin interalveolar septa (S) (Von Kossa, 400×).

extensive mineralization of alveolar septa (Figures 1b and 2b). Transverse sections of lung tissues of rats in the third group, which received 2 mg/kg of vitamin D_3 with 1000–9000 IU/kg per day of vitamin A, and of those in the fourth group, which received 2 mg/kg of vitamin D_3 and 15 mg/kg per day of vitamin K_1, revealed unremarkable results (Figures 1c and 2c, and Figures 1d and 2d, respectively).

3.1.2. Cardiac histopathological findings by a light microscope

Examination of cardiac sections from the control group showed a normal appearance of the myocardium, branching and anastomosis cardiac muscle fibers, acidophilic sarcoplasm, and central elongated vesicular nuclei (Figures 3a and 4a). However, cardiac tissues of rats in the second group, which received 2 mg/kg of vitamin D_3, showed a loss of normal cardiac architecture with fragmentation and degeneration of myocardial fibers; calcification of the epicardium, myocardium, endocardium, cardiac valves, and coronary arteries; and degeneration and fibrosis of the cardiac muscles with lymphomononuclear cell infiltration (Figures 3b and 4b). Cardiac sections of rats in the third group, which received 2 mg/kg of vitamin D_3 with 1000–9000 IU/kg per day of vitamin A, and of those in the fourth group, which received 2 mg/kg of vitamin D_3 and 15 mg/kg per day of vitamin K_1, showed the same normal appearance of the myocardium, branching and anastomosis cardiac muscle fibers with acidophilic sarcoplasm, and central elongated vesicular nuclei as those in the first group (Figures 3c and 4c, and Figures 3d and 4d, respectively).

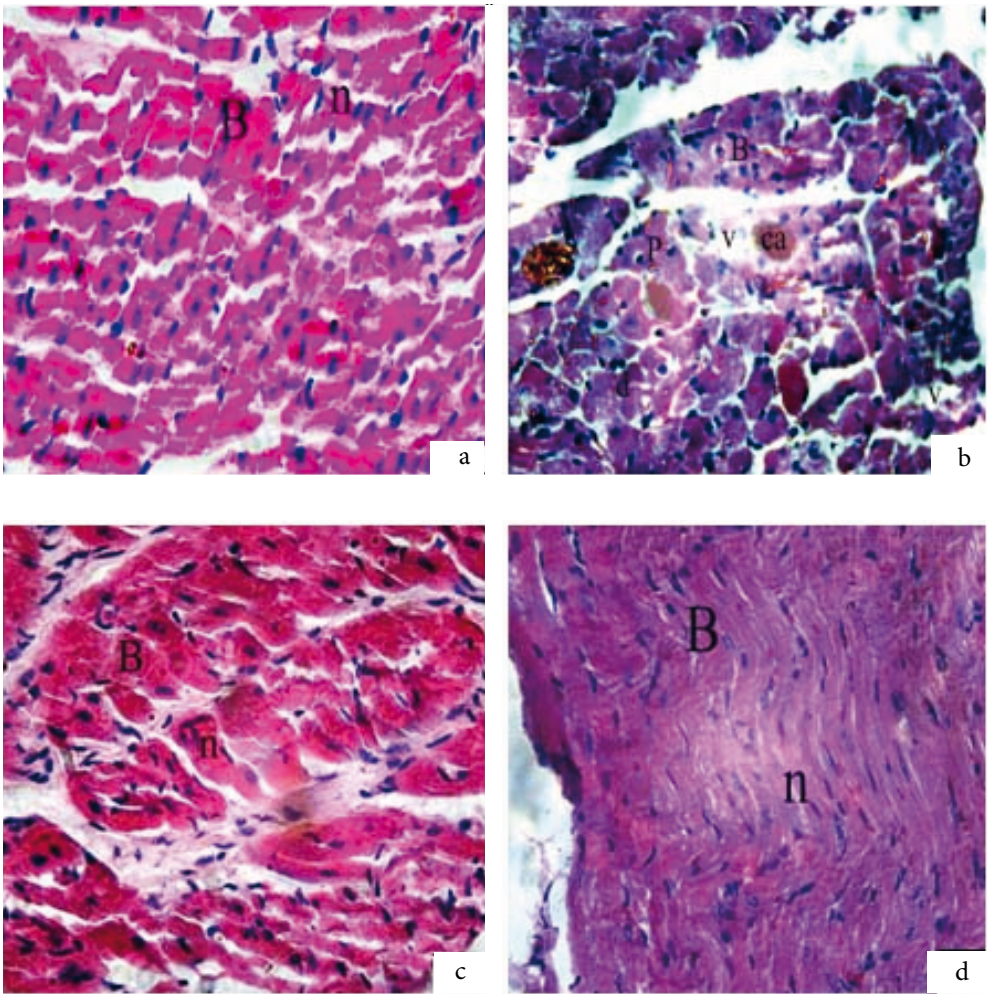

Figure 3. a) A photomicrograph of a transverse section in a control rat heart shows branching and anastomosis of cardiac muscle fibers (B) with acidophilic sarcoplasm and central elongated vesicular nuclei (n) (H&E, 400×); b) a photomicrograph of a transverse section in a rat heart from the second group shows loss of normal cardiac architecture, fragmentation and degeneration of the myocardial fibers (p), large-sized vacuoles (v) with acidophilic sarcoplasm, a degenerated area (d) with degenerated nuclei (n), and deposition of calcium (ca) (H&E, 400×); c) a photomicrograph of a transverse section in a rat heart from the third group shows normal branching and anastomosis of cardiac muscle fibers (B) with acidophilic sarcoplasm and central elongated vesicular nuclei (n) (H&E, 400×); d) a photomicrograph of a transverse section in a rat heart from the fourth group shows normal branching and anastomosis of cardiac muscle fibers (B) with acidophilic sarcoplasm and central elongated vesicular nuclei (n) (H&E, 400×).

3.1.3. Aortic histopathological findings by a light microscope

Examination of aortic sections from the control group showed normal components of the aortic wall (tunica intima, tunica media, and tunica adventitia) (Figures 5a and 6a). However, the aortic tissues of rats in the second group, which received 2 mg/kg of vitamin D_3, showed a loss of normal aortic structure, with fragmentation and degeneration of the aorta wall, and a wide separation of tunica media elastic fibers with calcium deposition (Figures 5b and 6b). Conversely, aortic sections of the third group, which received 2 mg/kg of vitamin D_3 with 1000–9000 IU/kg per day of vitamin A, and those of the fourth group, which received 2 mg/kg of vitamin D_3 and 15 mg/kg per day of vitamin K_1, showed no histological abnormalities of aortic wall components (Figures 5c and 6c, and Figures 5d and 6d, respectively).

3.1.4. Renal histopathological findings by a light microscope

Examination of renal sections from the control group revealed entirely normal histological features, as illustrated in Figures 7a and 8a. On the other hand, the renal sections in the second group, which received 2 mg/kg of vitamin D_3, showed shrinkage of some vascular glomeruli, tightness of the glomerular capsular space, degeneration of the epithelial lining of most renal tubules, and mineralization (nephrocalcinosis) of the cortex and medulla with calcified tubular epithelium (Figures 7b and 8b). In the

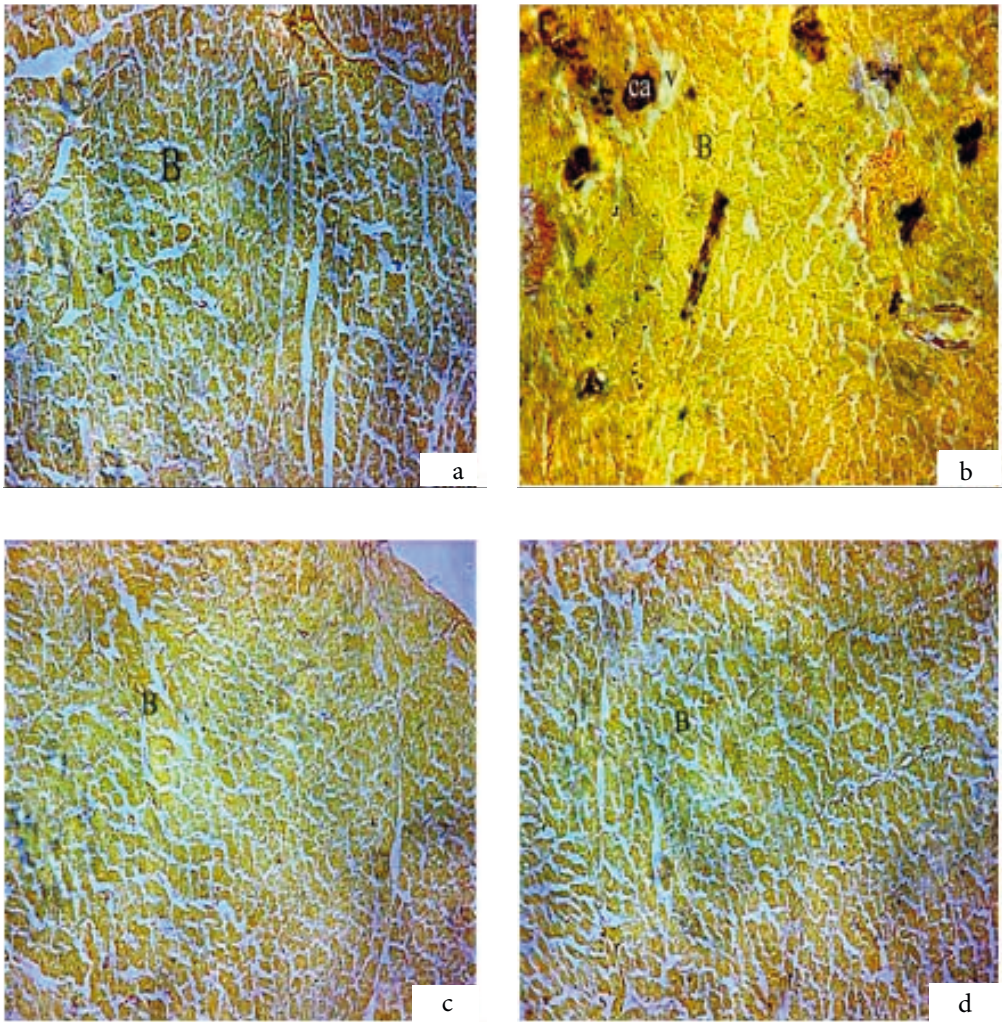

Figure 4. a) A photomicrograph of a transverse section in a control rat heart shows normal branching and anastomosis of cardiac muscle fibers (B) with acidophilic sarcoplasm and central elongated vesicular nuclei (n) (Von Kossa, 400×); b) a photomicrograph of a transverse section in a rat heart from the second group shows loss of normal cardiac architecture, fragmentation and degeneration of the myocardial fibers (B) with cavities (v), and deposition of calcification within muscle fibers (ca) (Von Kossa, 400×); c) a photomicrograph of a transverse section in a rat heart from the third group shows normal branching and anastomosis of cardiac muscle fibers (B) without calcium deposition (Von Kossa, 400×); d) a photomicrograph of a transverse section in a rat heart from the fourth group shows normal branching and anastomosis of cardiac muscle fibers (B) without calcium deposition (Von Kossa, 400×).

renal sections of the third group, which received 2 mg/kg of vitamin D_3 with 1000–9000 IU/kg per day of vitamin A, and in those of the fourth group, which received 2 mg/kg of vitamin D_3 and 15 mg/kg per day of vitamin K_1, no abnormal renal structures in both the cortex and the medulla were observed (Figures 7c and 8c, and Figures 7d and 8d, respectively).

3.1.5. Adrenal gland (suprarenal) histopathological findings by a light microscope

Examination of adrenal gland sections from the control group showed normal histological architecture (Figures 9a and 10a). However, suprarenal gland tissues of rats in the second group, which received 2 mg/kg of vitamin D_3, showed vacuolization of adrenal cortical cells and marked congestion of medulla and calcium deposition (Figures 9b and 10b). In the adrenal gland sections of the third group, which received 2 mg/kg of vitamin D_3 with 1000–9000 IU/kg per day of vitamin A, and in the adrenal gland sections of the fourth group, which received 2 mg/kg of vitamin D_3 and 15 mg/kg per day of vitamin K_1, no abnormal histological findings were observed (Figures 9c and 10c, and Figures 9d and 10d, respectively).

3.2. Biochemical findings

Table 1 represents the mean ± SD values of the renal function tests in the rats. The mean ± SD values of urea in the control group, second group, third group, and fourth group were 31.1 ± 8.49, 49.6 ± 1.46, 26.0 ± 2.07, and 34.4 ± 1.76, respectively. The value of F, which indicates the

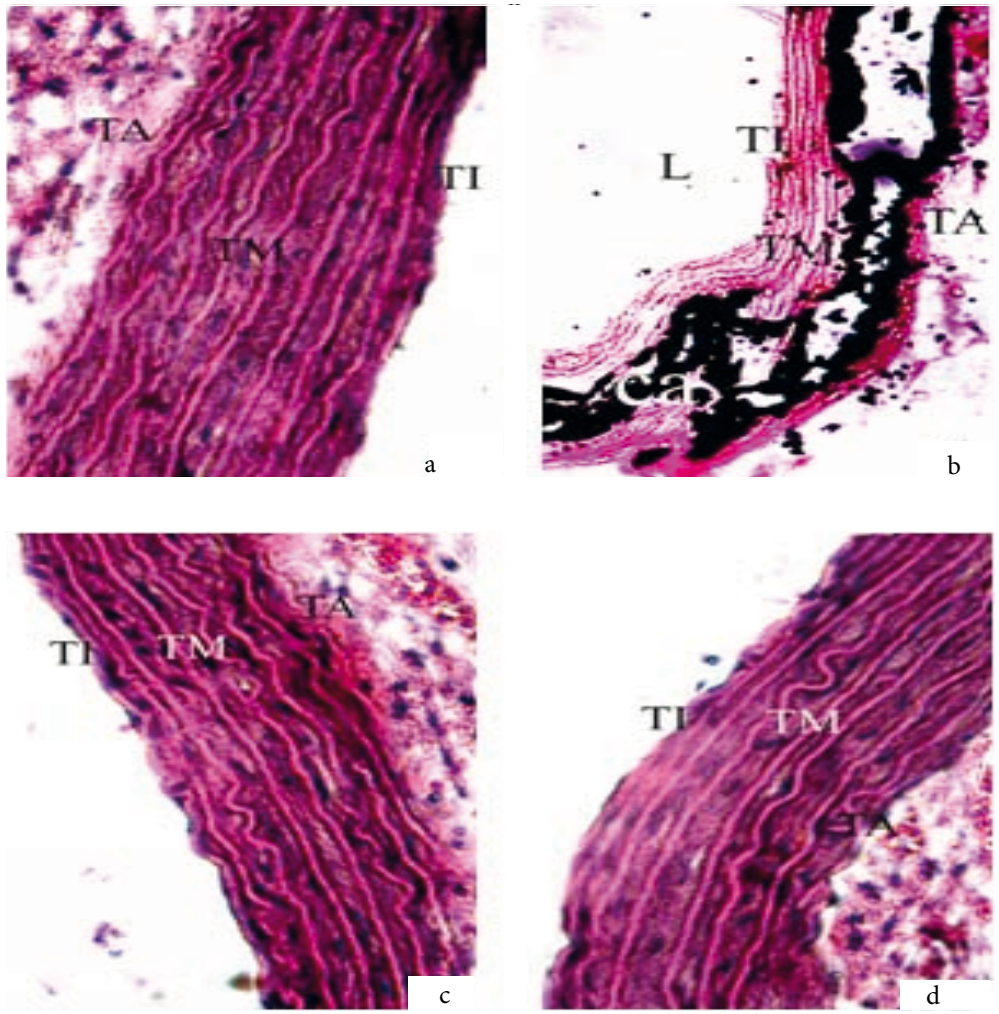

Figure 5. a) A photomicrograph of a transverse section in a control rat aorta shows normal alignment of aortic wall tunics (tunica intima "TI," tunica media "TM," and tunica adventitia "TA") (H&E, 400×); b) a photomicrograph of a transverse section in a rat aorta from the second group shows loss of normal aortic structure with fragmentation and degeneration of tunica intima (TI) and tunica adventitia (TA), and with tunica media (TM) calcification (ca) (H&E, 400×); c) a photomicrograph of a transverse section in a rat aorta from the third group shows normal alignment of the aortic wall tunics (tunica intima "TI," tunica media "TM," and tunica adventitia "TA") (H&E, 400×); d) a photomicrograph of a transverse section in a rat aorta from the fourth group shows normal alignment of the aortic wall tunics (tunica intima "TI," tunica media "TM," and tunica adventitia "TA") (H&E, 400×).

difference between groups, was 100.7 and significant, with $P < 0.001$. The mean ± SD values of creatinine in the control group, second group, third group, and fourth group were 0.91 ± 0.28, 0.66 ± 0.14, 0.70 ± 0.18, and 0.28 ± 0.11, respectively. The value of F, which indicates the difference between groups, was 36.38 and significant, with $P < 0.001$.

Table 2 represents the mean ± SD values of cardiac enzymes in the rats. The mean ± SD values of cTnI in the control group, second group, third group, and fourth group were 0.002 ± 0.001, 0.76 ± 0.014, 0.004 ± 0.0005, and 0.165 ± 0.164, respectively. The value of F, which indicates the difference between groups, was 22,302.43 and significant, with $P < 0.001$. The mean ± SD values of CK-MB in the control group, second group, third group, and fourth group were 1.90 ± 0.64, 22.1 ± 1.37, 1.45 ± 0.510, and 1.3 ± 0.47, respectively. The value of F, which indicates the difference between groups, was 3044.05 and significant, with $P < 0.001$. The mean ± SD values of myoglobin in the control group, second group, third group, and fourth group were 121.67 ± 0.60, 296.72 ± 1.33, 126.41 ± 0.229, and 129.35 ± 0.268, respectively. The value of F, which indicates the difference between groups, was 257,381.93 and significant, with $P < 0.001$.

Figure 11 represents the mean ± SD values of serum calcium in the rats. The mean ± SD values of serum calcium in the control group, second group (Vitamin D_3), third group (Vitamin D_3 and Vitamin A), and fourth group (Vitamin D_3 and Vitamin K_1) were 9.88 ± 0.19, 20.16 ± 0.57, 10.55 ± 0.28, and 10.47 ± 0.24, respectively. $P < 0.001$ was considered significant.

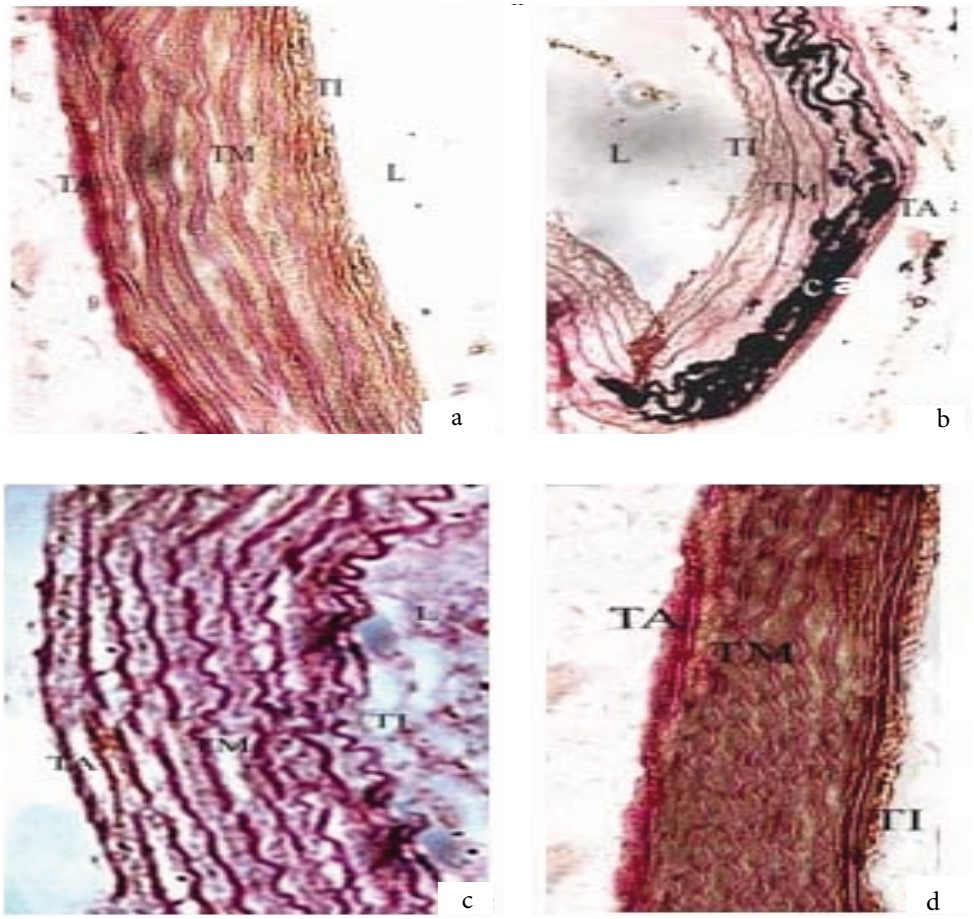

Figure 6. a) A photomicrograph of a transverse section in a control rat aorta shows normal alignment of the aortic wall tunics (tunica intima "TI," tunica media "TM," and tunica adventitia "TA") (Von Kossa, 400×); b) a photomicrograph of a transverse section in a rat aorta from the second group shows loss of normal aortic structure with fragmentation and degeneration of tunica intima (TI) and tunica adventitia (TA), and with calcium deposition (ca) between the elastic fibers of tunica media (TM) (Von Kossa, 400×); c) a photomicrograph of a transverse section in a rat aorta from the third group shows normal alignment of the aortic wall tunics (tunica intima "TI," tunica media "TM," and tunica adventitia "TA") (Von Kossa, 400×); d) a photomicrograph of a transverse section in a rat aorta from the fourth group shows normal alignment of the aortic wall tunics (tunica intima "TI," tunica media "TM," and tunica adventitia "TA") and lumen (L) (Von Kossa, 400×).

4. Discussion

Vitamin poisoning occurs over time or accidentally as a result of taking high doses of vitamins at once. Hypervitaminosis occurs with fat-soluble vitamins such as vitamin D because they accumulate in the body. Hypervitaminosis D_3 is usually iatrogenic because of the administration of high doses by physicians without clear diagnosis of vitamin insufficiency, although vitamin D_3 has a wide therapeutic index (15). Physicians recommend high daily doses of vitamin D_3 for many weeks as a treatment for chronic bone diseases (osteomalacia and osteoporosis) and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and psoriasis because they are associated with low blood levels of vitamin D metabolites; therefore, the incidence rate of hypervitaminosis D_3 may increase (16). Oncologists also recommend high daily doses of vitamin D_3 for a long time as prevention of epithelial malignancies such as breast and colon cancer and to reduce the recurrence and mortality of breast and ovarian cancer (17). Thus, the present study investigated the short-term toxicity of hypervitaminosis D_3 and how we can ameliorate this toxicity by using other vitamins such as vitamins A and K.

The present study showed a significant increase in the serum calcium level in the second group, which received a toxic dose of vitamin D_3, in comparison with the control group. Özkan et al. (18) showed that hypervitaminosis D_3 leads to an increase in bone resorption, and thus the occurrence of hypercalcemia and subsequent hypercalciuria. Our histopathological results supported the biochemical results because they referred to the presence of nephrocalcinosis.

The current study showed a significant increase in serum urea associated with a significant decrease in serum

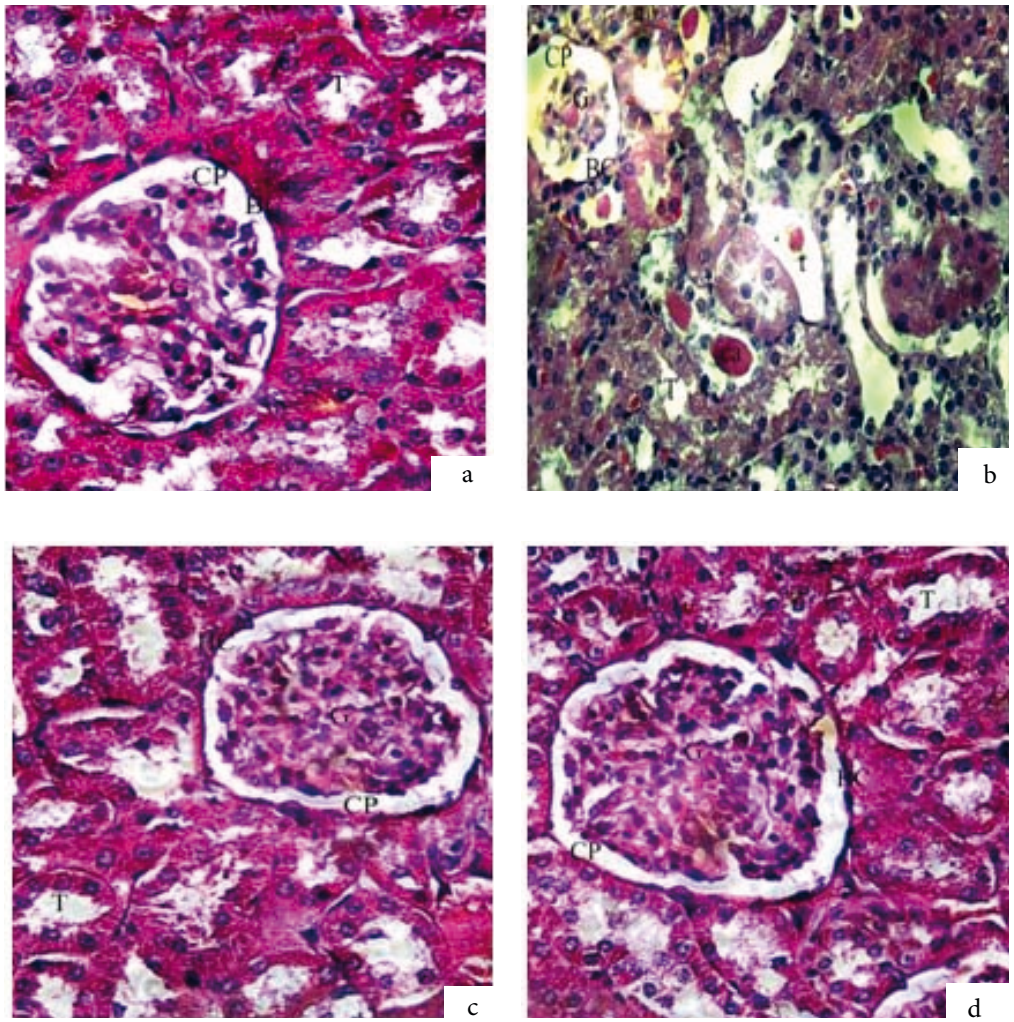

Figure 7. a) A photomicrograph of a section in a control rat kidney shows normal glomeruli (G) and glomerular capsular space (CP), with flat epithelium lining the Bowman's capsule (BC) and normal epithelial lining cells of the tubules (T) (H&E, 400×); b) a photomicrograph of a section in a rat kidney from the second group shows shrinking vascular glomeruli (G), a decrease in glomerular space (CP), with flat epithelium lining of Bowman's capsule (BC), degeneration of some tubule (T) epithelial lining, and complete destruction of epithelial cells of other tubules (t) (H&E, 400×); c) a photomicrograph of a section in a rat kidney from the third group shows normal glomeruli (G) and glomerular capsular space (CP), with flat epithelium lining the Bowman's capsule (BC) and normal epithelial lining cells of the tubules (T) (H&E, 400×); d) a photomicrograph of a section in a rat kidney from the fourth group shows normal glomeruli (G) and glomerular capsular space (CP), with flat epithelium lining the Bowman's capsule (BC) and normal epithelial lining cells of the tubules (T) (H&E, 400×).

creatinine in the second group, which received a toxic dose of vitamin D_3, in comparison with the control group. Ralston et al. (19) explained that low serum creatinine levels are due to high urine calcium creatinine, which is associated with an increase in the level of 25-hydroxy vitamin D. Hypercalcemia causes the accumulation of calcium phosphate crystals in soft tissues and then renal function impairment, leading to high serum urea, and this is consistent with results reported by Jones (20).

According to Koul et al. (21), the mechanism of vitamin D toxicity is due to an increased concentration of its metabolites reaching the vitamin D receptors in the target cells' nuclei, leading to exaggerated gene expression. The rise in plasma 25 (OH) D concentrations exceeding the vitamin D binding protein capacity leads to the entry of free 25(OH) D inside the cells, which has a direct effect on gene expression.

Our histopathological results revealed widespread calcification (calcium deposition) in all investigated organs (heart, kidney, lung, and adrenal glands) of the second group, which received a toxic dose of vitamin D_3. These histopathological findings (multiple organ mineralization) are consistent with results reported by Chavhan et al. (5) and by Kerr (22), who showed a rise in hydroxylated vitamin plasma levels according to the degree of vitamin overload and a prolongation of the hypercalcemia half-

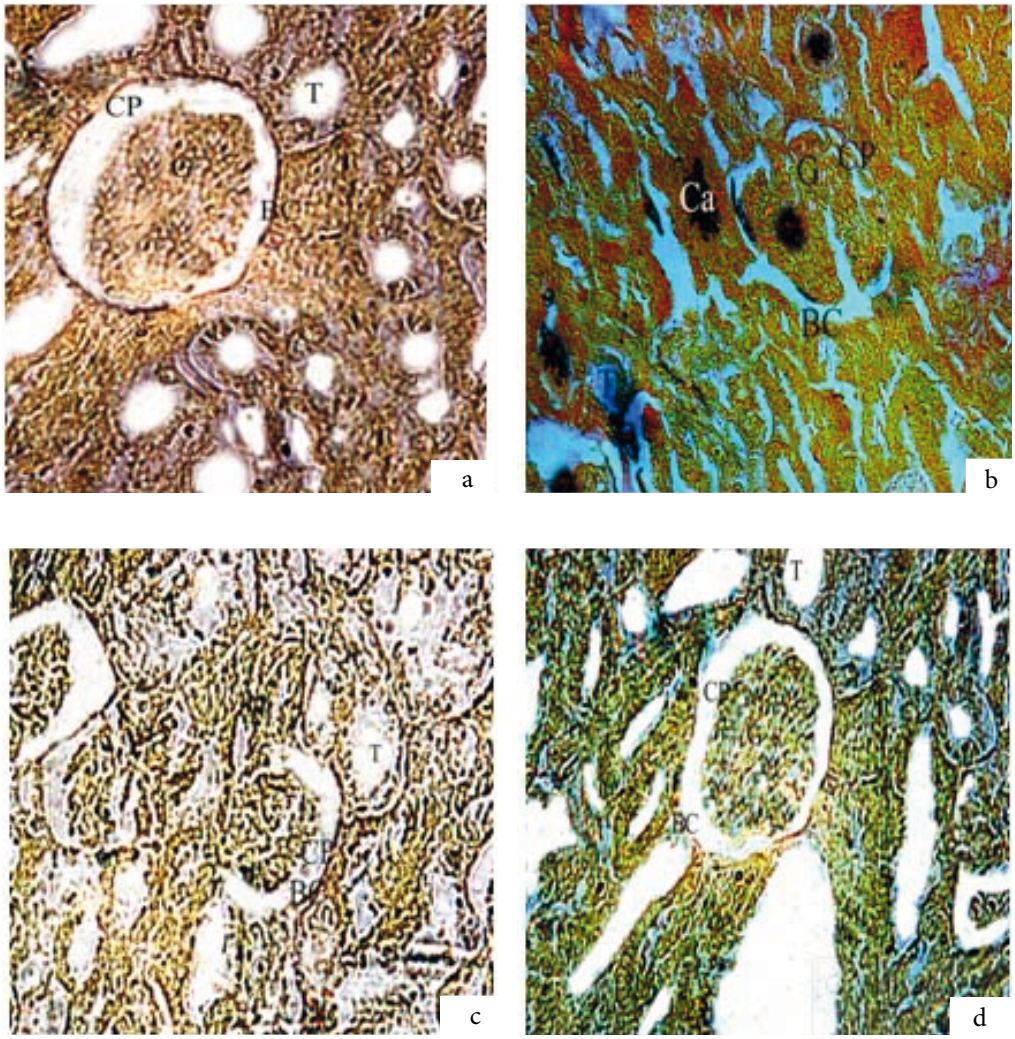

Figure 8. a) A photomicrograph of a section in a control rat kidney shows normal glomeruli (G) and glomerular capsular space (CP), with flat epithelium lining the Bowman's capsule (BC) and normal epithelial lining cells of the tubules (T) (Von Kossa, 400×); b) a photomicrograph of a section in a rat kidney from the second group shows shrinking vascular glomeruli (G), a decrease in glomerular space (CP), with flat epithelium lining of Bowman's capsule (BC), degeneration in some tubule (T) epithelial lining, and complete destruction of epithelial cells in other tubules (t) with deposition of calcium (ca) (Von Kossa, 400×); c) a photomicrograph of a section in a rat kidney from the third group shows normal glomeruli (G) and glomerular capsular space (CP), with flat epithelium lining the Bowman's capsule (BC) and normal epithelial lining cells in the tubules (T) (Von Kossa, 400×); d) a photomicrograph of a section in a rat kidney from the fourth group shows normal glomeruli (G) and glomerular capsular space (CP), with flat epithelium lining the Bowman's capsule (BC) and normal epithelial lining cells in the tubules (T) (Von Kossa, 400×).

life, which leads to rapid calcium deposition (calcification) in soft tissues. Drüeke and Massy (23) reported results that are consistent with those in the present study for aortic calcification; they showed that hypervitaminosis D_3 is associated with extensive arterial calcium phosphate deposition in the form of apatite crystals due to an increase in serum calcium and the formation of fetuin-A mineral complexes, which is associated with a decrease in free serum levels of fetuin-A. Kingma and Roy (24) are in agreement with our results and confirmed that hypervitaminosis D_3 induced arterial calcinosis.

Walentynowicz et al. (25) are also in agreement with the results of the current study, indicating that hypervitaminosis D_3 leads to toxic myocardial damage in the form of degeneration and calcification-inducing myocardial infarction due to the increase in the concentration of calcium in the blood and its subsequent deposition in the myocardium. The cardiac histopathological findings in the present study showed the cause of the significant increase of cardiac enzymes in the second group (which received a toxic dose of vitamin D_3). Cardiac enzymes such as creatine kinase (CK), its isoenzyme CK-MB, and cTnI are highly specific for the myocardium, and thus the gold standard for the detection of acute myocardial infarction in people and small animals according to Jeremias and Gibson (26) and O'Brien (27).

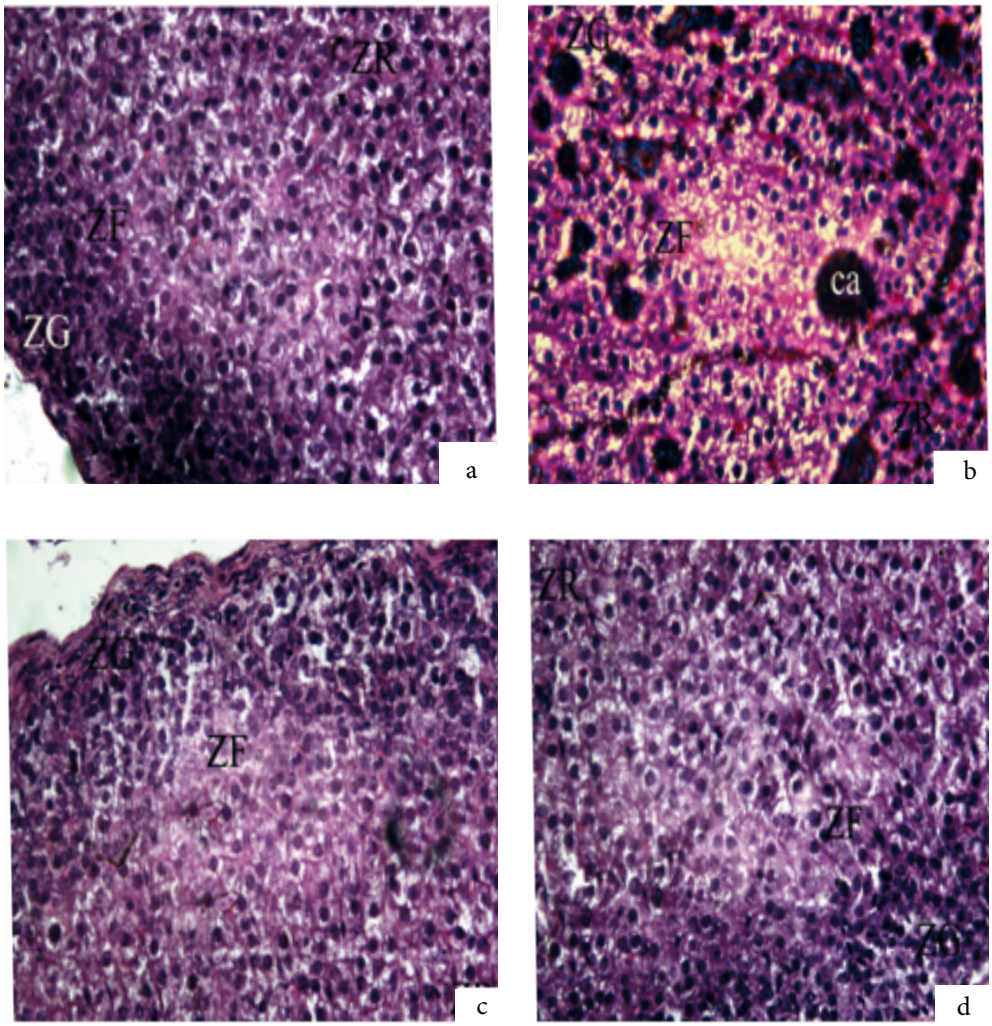

Figure 9. a) A photomicrograph of a section in a control rat adrenal gland shows normal zona glomerulosa (ZG) with cells in small clusters, zona fasciculate (ZF) with cells arranged in columns, and zona reticularis (ZR) with some unorganized cells (H&E, 400×); b) a photomicrograph of a section in a rat adrenal gland from the second group shows vacuolization of adrenal cortical cells and calcium deposition in each zone (ca) (H&E, 400×); c) a photomicrograph of a section in a rat adrenal gland from the third group shows normal zona glomerulosa (ZG) with cells in small clusters, zona fasciculate (ZF) with cells arranged in columns, and zona reticularis (ZR) with some unorganized cells (H&E, 400×); d) a photomicrograph of a section in a rat adrenal gland from the fourth group shows normal zona glomerulosa (ZG) with cells in small clusters, zona fasciculate (ZF) with cells arranged in columns, and zona reticularis (ZR) with some unorganized cells (H&E, 400×).

The current study showed significant differences for all parameters (serum calcium, renal function tests, and cardiac enzymes) in the third group, which received a toxic dose of vitamin D_3 with a therapeutic dose of vitamin A, and in the fourth group, which received a toxic dose of vitamin D_3 with a therapeutic dose of vitamin K_1, in comparison with the second group, which received a toxic dose of vitamin D_3 only. Furthermore, there was a significant difference for renal function tests and cardiac enzyme levels only in the fourth group in comparison with the third group, but the difference remained within normal limits. Our results showed markedly improved histopathological findings for all the investigated organs (kidney, heart, lung, adrenal gland, and aorta) in the third and fourth groups in comparison with the second group. These results confirm that vitamin K and vitamin A have the same protective role in the modulation of hypervitaminosis D_3 toxicity.

According to Adams and Pepping (28), vitamins A and K maintain healthy levels of matrix Gla proteins, which protect soft tissues from calcification. Vitamin K carboxylates osteocalcin, accumulates it in the extracellular matrix, and inhibits its release into the culture medium in a dose-dependent manner. The undercarboxylation of osteocalcin is likely to contribute to bone loss in hypervitaminosis D cases. The excessive stimulation

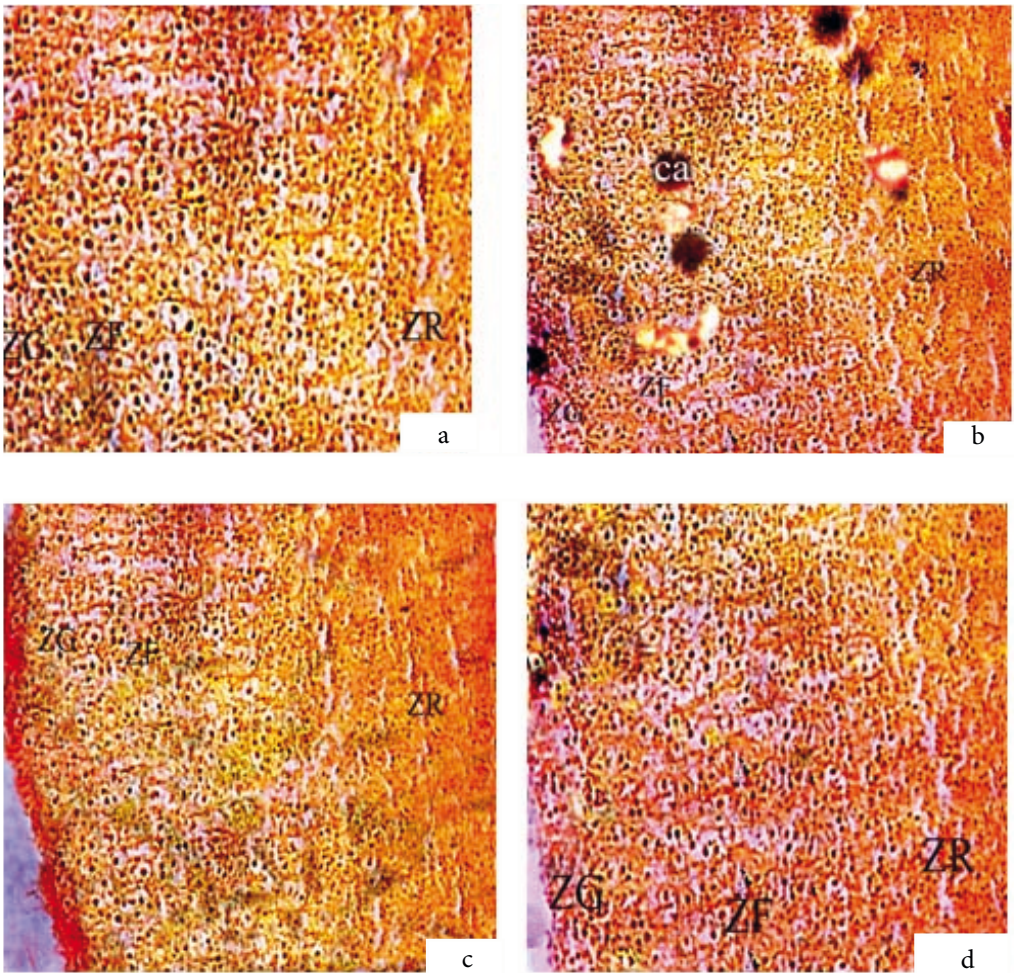

Figure 10. a) A photomicrograph of a section in a control rat adrenal gland shows normal zona glomerulosa (ZG) with cells in small clusters, zona fasciculate (ZF) with cells arranged in columns, and zona reticularis (ZR) with some unorganized cells (Von Kossa, 400×); b) a photomicrograph of a section in a rat adrenal gland from the second group shows vacuolization of adrenal cortical cells and calcium deposition in each zone (ca) (Von Kossa, 400×); c) a photomicrograph of a section in a rat adrenal gland from the third group shows normal zona glomerulosa (ZG) with cells in small clusters, zona fasciculate (ZF) with cells arranged in columns, and zona reticularis (ZR) with some unorganized cells (Von Kossa, 400×); d) a photomicrograph of a section in a rat adrenal gland from the fourth group shows normal zona glomerulosa (ZG) with cells in small clusters, zona fasciculate (ZF) with cells arranged in columns, and zona reticularis (ZR) with some unorganized cells (Von Kossa, 400×).

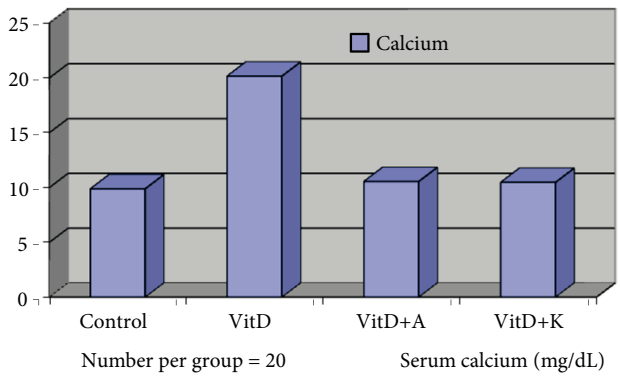

Figure 11. Comparison of the means ± SD of serum calcium among the different groups of rats.

Table 1. Comparison between the effects of vitamin K_1 and vitamin A on the means ± SD of renal function tests in hypervitaminosis D_3-intoxicated rats.

Parameter \ Group	First Mean ± SD	Second Mean ± SD	Third Mean ± SD	Fourth Mean ± SD	F
Urea (mg/dL)	31.1 ± 8.49	49.6 ± 1.46*	26.0 ± 2.07**	34.4 ± 1.76***	100.7
Creatinine (mg/dL)	0.91 ± 0.28	0.66 ± 0.14*	0.70 ± 0.18**	0.28 ± 0.11***	36.38

Number per group = 20. SD = standard deviation.
First group (control) received distilled water.
Second group received vitamin D_3 (2 mg/kg).
Third group received vitamin D_3 (2 mg/kg) with vitamin A (1000–9000 IU/kg per day).
Fourth group received vitamin D_3 (2 mg/kg) with vitamin K_1 (15 mg/kg per day).
* = P < 0.001 (significant difference in comparison with the control group).
** = P < 0.001 (significant difference in comparison with the second group).
*** = P < 0.001 (significant difference in comparison with the third group).

Table 2. Comparison between the effects of vitamin K_1 and vitamin A on the means ± SD of cardiac enzyme levels in hypervitaminosis D_3-intoxicated rats.

Parameter \ Group	First Mean ± SD	Second Mean ± SD	Third Mean ± SD	Fourth Mean ± SD	F
Troponin-1 (ng/mL)	0.002 ± 0.001	0.76 ± 0.014*	0.004 ± 0.0005**	0.165 ± 0.164***	22,302.43
CK-MB (ng/mL)	1.90 ± 0.64	22.1 ± 1.37*	1.45 ± 0.510**	1.3 ± 0.47***	3044.05
Myoglobin (ng/mL)	121.67 ± 0.60	296.72 ± 1.33*	126.41 ± 0.229**	129.35 ± 0.268***	257,381.93

Number per group = 20. SD = standard deviation. CK-MB = Creatine kinase-MB.
First group (control) received distilled water.
Second group received vitamin D_3 (2 mg/kg).
Third group received vitamin D_3 (2 mg/kg) with vitamin A (1000–9000 IU/kg per day).
Fourth group received vitamin D_3 (2 mg/kg) with vitamin K_1 (15 mg/kg per day).
* = P < 0.001 (significant difference in comparison with the control group).
** = P < 0.001 (significant difference in comparison with the second group).
*** = P < 0.001 (significant difference in comparison with the third group).

of matrix Gla proteins and other vitamin K-dependent proteins could causally contribute to the observed toxicity. This is inconsistent with the findings of Fu et al. (29), who reported that vitamin K increased bone mineralization and decreased bone resorption through its role as an enzyme cofactor or as independent from ỹ-carboxylation mechanisms. Ferland (30) indicated that the mechanism of vitamin D_3 toxicity modulation by vitamin K administration may be due to the biological activity of vitamin K-dependent proteins, which are synthesized in the liver and then secreted into circulation, where they depend on the normal complement of Gla residues, which are efficient chelators of calcium ions counteracting hypercalcemia induced by hypervitaminosis D_3. Our results are consistent with those reported by El-Morsy et al. (31), who referred to the protective effect of vitamin K in hypervitaminosis D_3 and the lack of any known toxic adverse effects from the use of large amounts of vitamin K as a therapeutic agent, even 500 times the recommended daily allowance for a long period, with the exception of some rare hypersensitivity reactions that may occur when given intravenously (32).

According to Penniston and Tanumihardjo (33), vitamin A modulates vitamin D_3 toxicity because of an antagonism between vitamins A and D at the receptor level and an interaction with calcium regulating hormones such as parathyroid hormone (34), whereas the receptors for retinoic acid are located on osteoblasts and osteoclasts (35). Therefore, the therapeutic doses of vitamin A are given safely for a long period to ameliorate the manifestations of hypervitaminosis D_3, whereas a low incidence of transient toxic effects of vitamin A may be expected and tolerated (36,37).

Masterjohn (38) referred to the protective effect of vitamin A in vitamin D_3 toxicity and its ability to downregulate matrix Gla proteins in soft tissues, and thus prevent calcification in renal tissue; however, a limited effect on lung and heart tissues was reported in that study. This is in contrast with our results, which confirmed the ability of vitamin A to prevent tissue calcification in all organs without exception.

Price et al. (39) showed that vitamin D_3 toxicity leads to an increase in the matrix Gla protein expression, depending on tissue type, in correlation with the degree of calcification, and coinciding simultaneously with hypercalcemia; however, they reported that the restoration of normal levels of matrix Gla proteins was not associated with a reduction in the serum calcium level because the increase in matrix Gla protein levels was not a reaction to serum calcium elevation. This is in contrast with our results, which indicated widespread calcification in different organs apart from their tissue type correlating with hypercalcemia, and thus the improvement of calcification is associated with a decrease in serum calcium levels.

Hypervitaminosis D_3 leads to toxic manifestations representing an increase in serum calcium levels, cardiac enzyme elevations, disturbances in renal function tests, and histopathological changes in the kidney, heart, lung, aorta, and adrenal gland in rats. The administration of vitamin A or K with a toxic dose of vitamin D_3 modulates short-term toxicity manifestations induced by hypervitaminosis D_3.

According to the results of the present study, the use of high doses of vitamin D_3 without monitoring should be avoided. If there is a need for high dosages of vitamin D_3, protective agents such as vitamin K or vitamin A should be used because they modulate vitamin D_3 toxicity with the same efficacy. Further research in humans is recommended in order to verify our results.

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