The Effects of Hypervitaminosis D in Rats on Histology and Weights of Some Immune System Organs and Organs Prone to Calcification

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ABSTRACT

Vitamin D is essential for overall health, wellbeing, and the immune system. Hypervitaminosis D leads to many deleterious effects and increased mortality. This study was done to evaluate the effects of vitamin D toxic doses on histology and weight indices of some main immune system organs (spleen and thymus), and some organs that are prone to calcification due to hypervitaminosis D (liver, kidney, and heart) in rats. Thirty-five adult male Wistar rats were randomly assigned to four groups receiving water or vitamin D by oral gavage. The control group (8 rats) received distilled deionized water for 22 days. The experimental groups were low dose (LD, 9 rats, 1,500 IU of vitamin/rat/day for 21 days), intermediate dose (ID, 9 rats, 3,000 IU/rat/day for 22 days), and high dose (HD, 9 rats, 6,750 IU/rat/day for 22 days). No other studies used such high doses on rats. Compared to the control group, the mean liver and kidney indices for the ID group were both significantly lower, while the mean heart index for the LD group was significantly higher. The spleen and thymus indices were not significantly different. Histopathology of the organs showed minimal changes for the LD group while the ID and HD groups had focal degenerative changes in the liver and more so in the kidney. Lymphocytes were decreased in both the spleen and thymus parenchyma. In conclusion, the higher doses of vitamin D had toxic effects on the organs studied and thus affected the immune system and health in general.

Key Words: Vitamin D, Hypervitaminosis D, Immune System Organs, Calcification, Thymus, Spleen, Kidney, Liver, Heart, Histology.

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INTRODUCTION

Proper nutrition is essential for health and proper functioning of all systems in the body, including the immune system. Excess or deficit of many nutrients may lead to detrimental effects and increased susceptibility to many diseases due to the effects on health and different systems of the body [1-3]. Micronutrient deficiencies are widespread worldwide, especially in low-income countries but also in developed ones, with more than 2 billion people being affected [3]. They are the leading causes of chronic diseases and increased morbidity and mortality. Vitamin D deficiency is one of the most prevalent nutrition deficiencies worldwide, with 1 billion people of all ages and genders being affected [4].

Vitamin D, a fat-soluble vitamin, may be obtained from food, sun exposure, and supplements. Vitamin D has two active forms in the body, which are vitamin D_2 (ergocalciferol), which comes from dietary sources, and vitamin D_3 (cholecalciferol), which results from the activation of 7-dehydrocholesterol by sun exposure. The vitamin is present in the body in a biologically inert form that must be activated by undergoing two hydroxylations, with the first occurring in the liver and the second in the kidney. Therefore, both the liver and kidney are important for the activation of vitamin D. In addition, it is known that excess vitamin D is stored in the liver and fat tissue

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of the body.

Vitamin D is important for general health, calcium and phosphate metabolism, and bone health. It also reduces the risk of diabetes, rheumatoid arthritis, some cancers, heart diseases, and infectious diseases. In addition, it has been shown to affect both the innate and acquired immunities [5, 6]. The multiple functions of vitamin D in the body are mediated by binding of the activated vitamin D to the vitamin D receptor, which is present in almost all cells and tissues of the body, including immune system cells, enabling them to respond to the active form of the vitamin [7, 8].

Vitamin D deficiency may lead to rickets in children, and increased risk of fractures, osteopenia, osteoporosis, osteomalacia, and muscle weakness in adults. It is also linked to cardiovascular diseases, cancer, infectious diseases, autoimmune diseases, inflammatory diseases, hypertension, obesity, and even cognitive impairment and depression [6, 9-12]. Therefore, many people take vitamin D supplements, and many countries supplement some foods with vitamin D to circumvent this problem. This may result in vitamin D overdose, also termed hypervitaminosis or intoxication, although it is very rare [13, 14]. In humans and animals, vitamin D hypervitaminosis leads to increased calcium concentration in the blood leading to the calcification of soft tissues and organs, such as the liver, kidney, and heart, and overcalcification of bones [15, 16]. Vitamin D toxicity in rats leads to the symptoms that are similar to humans, such as ruffled coat, dullness, anorexia, cachexia, progressive weight loss, rigidity of limbs, ataxic movement, difficulty in respiration, diarrhea, decreased food and water consumption, epistaxis, shivering, subnormal body temperature, nervous signs, and possibly death [17].

There are not many studies on the effects of vitamin D overdose on humans and animals, probably due to the rarity of hypervitaminosis D. Therefore, this study examined the effects of high vitamin D levels, that lead to hypervitaminosis D, on clinical signs, histopathology and weights of rat organs involved in the immune response (thymus and spleen), activation and storage of vitamin D (kidney and liver), and calcification (kidney, liver and heart). The very high vitamin D doses used in this study have not been previously used by other researchers and thus the study presented new and novel findings.

MATERIALS AND METHODS

Experimental animals

A total of 35 adult male Wistar rats aged about 8 weeks and weighing 201-278 g were used in this study. All rats were supplied by and housed at King Fahd Medical Research Center, Jeddah, Saudi Arabia. The rats were housed at room temperature (25 °C), and were exposed to artificial light during working hours and to the natural light-dark cycle afterwards. They were allowed the free access to food and water during the entire experimental period. All rats received the same diet (Grain Silos and Flour Mills Organization, Jeddah, Saudi Arabia), containing 20% crude protein, 4% crude fat, 3.50% crude fiber, 6% ash, 0.50% salt, 1% calcium, 0.60% phosphorus, 20 IU/g vitamin A, 2.20 IU/g vitamin D, and 70 IU/g vitamin E. This study was approved by the university and it was in compliance with the regulations on ethical treatment of the laboratory animals.

Categorization and vitamin administration

The rats were randomly divided into a control group of 8 rats and three experimental groups with 9 rats each. Liquid vitamin D_3 (cholecalciferol, 4,500 IU/ml, in 65.36% ethanol by volume) (Novartis Pharma AG, Basle, Switzerland) was diluted with distilled deionized water as needed. The control and experimental rats received their doses, as described below, by oral gavage at the same time daily, except on weekends (three weekends). The control group received distilled deionized water at 1 ml/rat for 22 days, and they were dissected on the 25th day. The low dose group (LD) received vitamin D at a concentration of 1,500 IU/rat for 27 days, which equaled 21 doses and they were dissected on the 28th day. The intermediate dose group (ID) received vitamin D at a concentration of 3,000 IU/rat for 28 days, leading to the administration of 22 doses, and their dissection was performed on the 29th day. Finally, the high dose group (HD) received vitamin D at 6,750 IU/rat for 28 days, leading to 22 doses, and they were also dissected on the 29th day.

Organs harvest

At the end of the experimental period, the rats were anesthetized by ether and euthanized by cervical dislocation and quickly dissected. The abdomen and chest were opened, and then the heart was perfused via the left ventricle with saline followed by 10% neutral buffered formalin to ensure good fixation of all organs. The liver, both kidneys, heart, spleen and thymus gland were excised, weighed and placed in 10% formalin in phosphate buffered saline (PBS) (Sigma Chemical Company, Saint Louis, USA) for fixation and storage at room temperature for two days for histopathological examination.

Calculation of organ indices

Organs indices were calculated by dividing the weight of the organ by the final body weight of the rat and then multiplying by 100, as shown by the following formula:

Organ index (%) = Organ weight (g) / Final body weight (g) × 100

Histological examination

One to two days after excising the organs, the liver was cut into small slices, the kidney was cut in a sagittal or transverse planes, and the parts of the left and right ventricles of the heart were trimmed. The organs' segments were then dehydrated in ascending grades of ethyl alcohol, starting at 70% of ethyl alcohol in distilled water and ending with 100% alcohol. Finally, once the water had been replaced by 100% ethyl alcohol, the tissues were cleared in xylene. Subsequently, they were placed in melted paraffin in an oven at 55 °C and, finally, embedded in cassettes. After cooling, the tissues were hardened, and they were sliced at 4 microns thickness using a microtome. The slices were passed through xylene, and then decreasing grades of ethyl alcohol (100% to 70%) and ending with 100% water. Finally, the tissues were stained with hematoxylin and eosin stains, and subsequently were examined and photographed using a microscope digital camera (Olympus BX51, Olympus Company, Tokyo, Japan) and the Olympus cellSens imaging software (Olympus Company, Pennsylvania, USA).

Statistical analysis

The Statistics Package for Social Sciences (SPSS) program, version 20, was used to analyze the data. The data were expressed as arithmetic mean, and standard deviation of the mean (± SD) or standard error (± SE). The differences between groups were analyzed using the one-way analysis of variance (ANOVA) test while the least significant difference (LSD) test was used for the differences between groups. P-values were calculated and used to indicate the significance between the groups as follows: highly significant ($P < 0.01$), significant ($0.01 \leq P \leq 0.05$), or none significant ($P > 0.05$) difference.

RESULTS

Observed clinical signs

In the first week of the experiment, there were no significant changes in the activity of rats in all experimental groups (LD, ID and HD groups). During the second week, rats of the LD and ID groups displayed decreased activity, while nervous signs, such as aimless running and rolling, were observed in rats of the HD group. During the third week and thereafter, rats in the experimental groups were less active than usual. On the 15^{th} and 18^{th} days, two rats from the HD group were found dead. Before dying, these rats were less active, weak and they showed difficulty in movement and respiration, in addition to shivering and epistaxis.

Organ indices for the rats

Using the one-way ANOVA test to compare the organ indices, there were no significant differences in the mean indices of the spleen and thymus between the four groups (Table 1). On the other hand, there were highly significant differences in the mean indices of the liver, kidney, and heart.

Using the LSD test for the comparisons between the groups (Table 2), the mean liver index for the ID group was significantly lower compared to the mean liver index for the control group. In addition, the mean liver index for the ID group was highly and significantly lower than the mean liver indices for the LD and HD groups, respectively. As for the kidney indices, the mean index for the ID group was highly and significantly lower compared to the mean index of control group. In addition, the mean kidney indices for the ID and HD groups were highly and significantly lower, respectively, compared to the mean LD group index. The mean heart index for the LD group was significantly higher compared to the mean index for the control group. Additionally, the mean heart indices for the ID and HD groups were highly and significantly lower than the mean index of LD group. All the other comparisons between the groups for the mean indices for the liver, kidney, and heart showed no significant differences.

Table 1: Descriptive statistics and test of significance for mean organ indices for the groups, using the one-way ANOVA test.

Organ	Group	Organ index (%)			± SD	P-value
		Maximum	Minimum	Mean		
Liver	Control	4.92	3.25	4.11	0.53	0.003^{HS}
	LD	4.93	3.65	4.39	0.40	
	ID	4.02	2.75	3.46	0.48	
	HD	4.55	3.31	4.07	0.52	
Spleen	Control	0.48	0.27	0.35	0.08	0.668^{NS}
	LD	0.46	0.30	0.36	0.05	
	ID	0.46	0.24	0.33	0.06	
	HD	0.44	0.28	0.36	0.06	
Kidney	Control	1.06	0.69	0.89	0.12	0.002^{HS}
	LD	1.02	0.70	0.92	0.09	
	ID	0.92	0.62	0.74	0.10	
	HD	0.85	0.73	0.80	0.04	
Heart	Control	0.42	0.29	0.37	0.04	0.002^{HS}
	LD	0.48	0.33	0.41	0.04	
	ID	0.39	0.29	0.33	0.03	

	HD	0.39	0.30	0.35	0.03	
Thymus	Control	0.22	0.10	0.12	0.04	0.337^{NS}
	LD	0.18	0.11	0.13	0.02	
	ID	0.16	0.08	0.10	0.03	
	HD	0.20	0.08	0.12	0.04	

SD: Standard deviation, LD: Low dose, ID: Intermediate dose, HD: High dose
HS: Highly significant (P < 0.01), NS: Not Significant (P > 0.05)

Table 2: Multiple comparisons between groups for mean organ indices, using the LSD test.

Organs	Group (X)	Group (Y)	Mean Difference (X–Y)	± SE	P-value
Liver	Control	LD	-0.28	0.23	0.233^{NS}
		ID	0.60	0.23	0.010^{S}
		HD	0.04	0.25	0.872^{NS}
	LD	ID	0.93	0.22	0.000^{HS}
		HD	0.32	0.24	0.190^{NS}
	ID	HD	-0.60	0.24	0.019^{S}
Kidney	Control	LD	-0.02	0.04	0.596^{NS}
		ID	0.15	0.04	0.003^{HS}
		HD	0.09	0.05	0.071^{NS}
	LD	ID	0.18	0.04	0.001^{HS}
		HD	0.12	0.05	0.021^{S}
	ID	HD	-0.05	0.05	0.255^{NS}
Heart	Control	LD	-0.04	0.02	0.022^{S}
		ID	0.03	0.02	0.120^{NS}
		HD	0.01	0.02	0.620^{NS}
	LD	ID	0.08	0.01	0.000^{HS}
		HD	0.06	0.02	0.008^{HS}
	ID	HD	-0.02	0.02	0.312^{NS}

SE: Standard error, LD: Low dose, ID: Intermediate dose, HD: High dose
HS: Highly significant (P < 0.01), S: Significant (0.01 ≤ P ≤ 0.05), NS: Not Significant (P > 0.05).

Effects of vitamin D on histology of immune organs

Histology of control rat thymus

The histological examination of control rat thymuses (Figures 1 and 2) revealed a normal morphology that consisted of interconnected lobules separated by scanty loose connective tissue. The cortex of each lobule was heavily populated with thymocytes, and a lower amount of reticulocytes. On the other hand, the medulla contained a smaller cell population of thymocytes and a larger number of reticulocytes compared to the cortex. Blood vessels were clearly seen in both the cortex and medulla.

Histology of supplemented rat thymus

Histological examination showed that the low dose of vitamin D did not affect many histological features of the thymus gland, as shown in Figures 1 and 2. However, as the dose increased (ID and HD), the depletion of the thymocyte cell population in both the cortex and medulla was observed with the effect being higher in rats receiving the high dose. In addition, reticulocytes, with active large nuclei, became more prominent especially within the medullary regions of lobules.

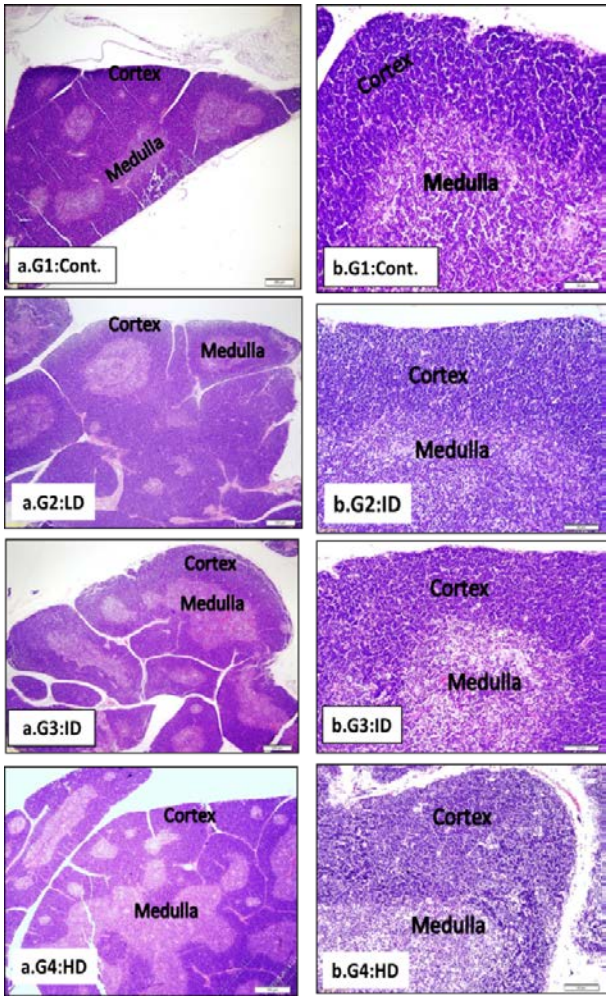

Fig. 1 Low (a) and high (b) magnification of sections of the thymus from both control and vitamin D supplemented rats showing: (G1: Control) a normal cell population in both the cortex and medulla. (G2: LD) minimal change in cell population. (G3: ID) moderate depletion of thymocytes in both the cortex and medulla. (G4: HD) focal regions of marked cell depletion in the cortex with lightly stained medulla due to a lower thymocyte population.

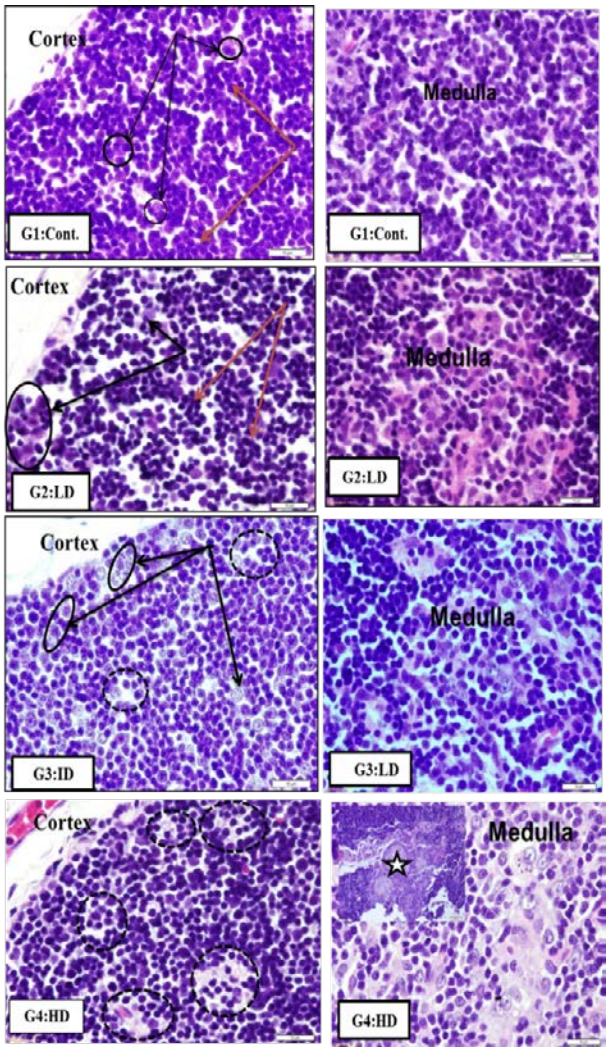

Fig. 2: Higher magnification of the cortex and medulla sections of thymic lobules from the control and vitamin D supplemented rats. Note that with increasing the dose of vitamin D, there was a decrease in the population of thymocytes population, which were cells with dark nuclei and scanty cytoplasm (red arrows) (G1 and G2, left side), and an increase in reticulocytes, which were the cells with lightly stained cytoplasm and large rounded vesicular nuclei (black arrows) (G1-G3, left side). Cell nests could be observed (dotted circle) (G3 and G4, left side) especially in the HD group (G4, left side). In some thymuses of the HD group, reticulocytes formed cell clusters (white star) (G4, right side).

Histology of control rat spleen

The spleen of control rats was covered by a thin fibromuscular capsule, with the parenchyma clearly differentiated into red and white pulp (Figure 3). The white pulp was characterized by the presence of the central arteriole, which was surrounded by lymphocytes that could not be identified to be T or B lymphocytes. The lymphoid nodules had ill-defined narrow germinal centers. Red pulp consisting of splenic blood sinusoids together with splenic lymphatic cell cords could be seen between the white pulp tissue.

Histology of supplemented rat spleen

Rats given vitamin D at all doses showed mild focal thickening of the splenic capsule and the wall of the central arteriole. The enlargement of white pulp was observed with increased vitamin D dose. Lymphoid follicles showed increases in the area of germinal centers. These changes were dose dependent, and they were evident mainly in spleens of the high dose group, as shown in Figure 3.

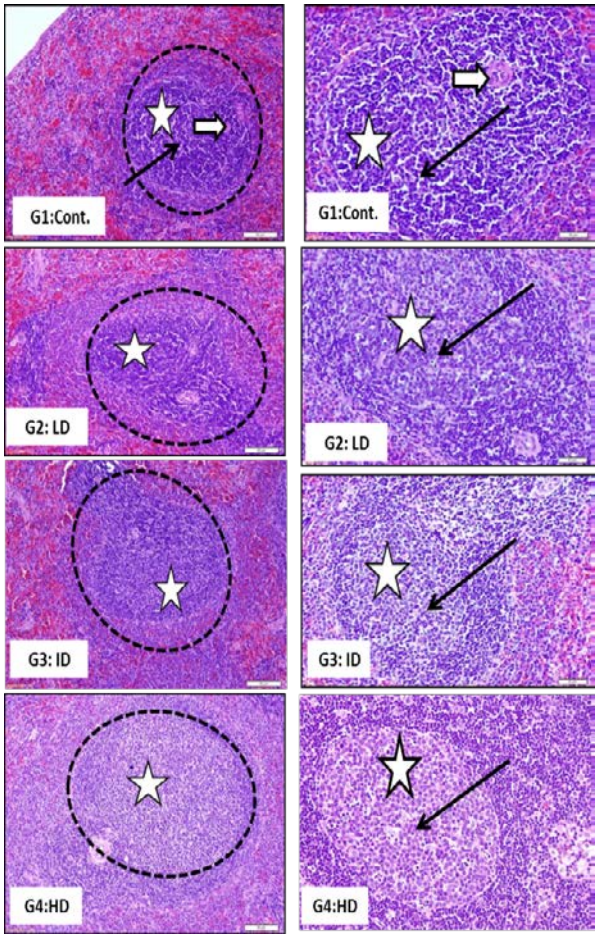

Fig. 3: Sections of supplemented rat spleens showed that as the vitamin D dose increased, there was a progressive enlargement of the white pulp (dotted circle and white star) (G2-G4, left side) with increasing size of the active germinal centers (black arrows) (G2-G4, right side) that looked lighter with an increased number of cells with cell debris (nest cells). This was evident mostly in the high dose (G4: HD).

Effects of vitamin D on vital organs

Histology of control rat liver

Control rat liver showed normal histology with a thin capsule and an ill-defined parenchyma of the lobules

identified only by the presence of central veins (CV) and peripheral portal areas. The portal area contained branches of the portal vein (PV) and hepatic artery (HA), and one or two bile ducts (BD). The higher magnification of the control liver showed hepatocytes arranged as plates or cords radiating from the central vein (CV) and separated by blood sinusoids lined by thin squamous epithelium. Von Kupffer phagocytes were few with their nuclei occasionally seen projecting to the sinusoidal lumen. The triad of the portal area might be clearly seen in the tissue sections.

Histology of supplemented rat liver

In vitamin D supplemented rats (Figures 4 and 5), as the vitamin dose increased, there was greater focal thickening of the capsule and more underlying cells showing inactive dark nuclei. In livers of rats taking the LD, there were mild changes in liver tissue with a few cells showing dark cytoplasm and small dark nuclei. Other cells had normal nuclei with prominent nucleoli. Von Kupffer cell nuclei were prominent. In the ID liver, the clusters of hepatocytes with dark stained cytoplasm and small dark nuclei (which are signs of apoptosis) were observed. Those cells looked shrunken and separated from the surrounding normal hepatocytes with increasing dose of vitamin D. In the HD liver, there was an increased amount of dark apoptotic cells, compared to the LD and ID groups. Near the portal area, the dilation of portal vein (PV) and inflammatory cells around a bile duct increased with increasing the doses of vitamin D. The congestion of portal vessels was also observed as shown in Figures 4 and 5.

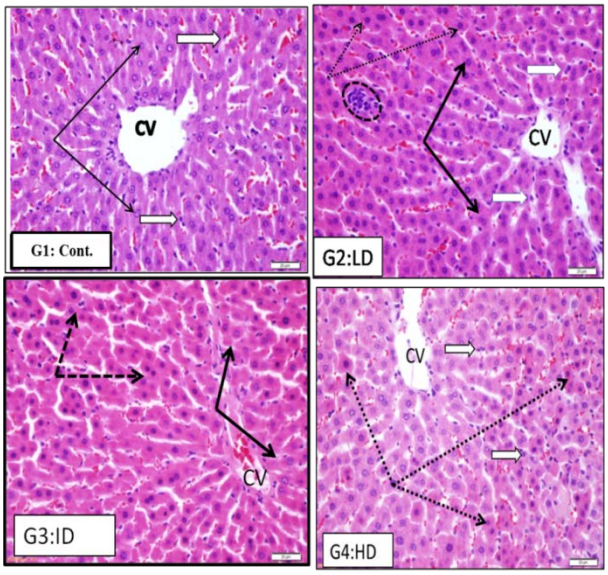

Fig. 4: Sections from rat liver of different groups near the central vein (CV). (G1: Cont.) Normal hepatocytes (black arrows) and related sinusoids (white arrows). (G2: LD) Normal hepatocytes (black arrows) with a few scattered cells having dark cytoplasm and small dark nuclei indicating apoptotic changes (dotted arrows). Note the presence of a few small foci of degenerated or inflammatory cells (dotted circle). (G3: ID) Showing a moderate increase of apoptotic cells (dotted arrows) and Von Kupffer cell nuclei are prominent (black arrows). (G4: HD) Numerous dark apoptotic cells that looked separated from adjacent normal hepatocytes (dotted arrows). Sinusoids showed prominent Von Kupffer cells (white arrows).

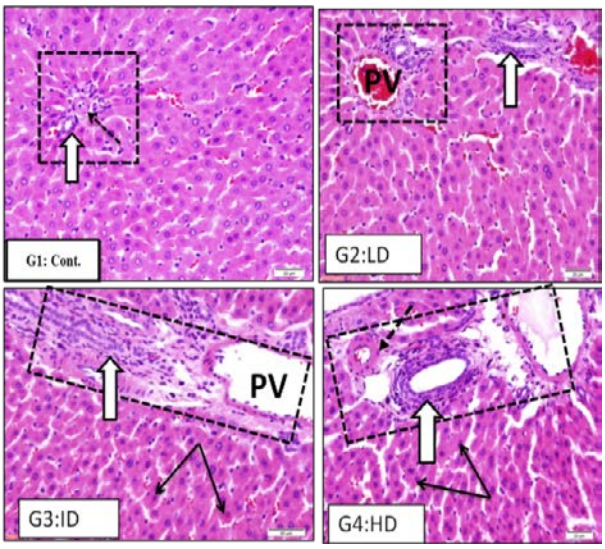

Fig. 5: Sections from rat liver near a portal region (dotted square) showing: (G1: Cont.) normal portal area contents, bile duct (white arrow), and hepatic artery (dotted arrow). (G2: LD) shows the congestion of the portal veins (PV) and a mild increase in the inflammatory cells around a bile duct (BD) (white arrow). (G3: ID) shows the dilation of the portal vein (PV) and inflammatory cells around a bile duct (white arrow). The nearby hepatocytes showed inactive dark nuclei (black arrows). (G4: HD) shows the marked dilation of the portal vein (PV) and thickening of bile duct wall with more inflammatory cells (white arrow). Nearby hepatocytes showed inactive dark cytoplasm and nuclei.

Histology of control rat kidney

The kidney of the control rats (Figure 6) showed a normal structure with a thin capsule and an outer cortex characterized by the presence of renal corpuscles that were well organized. The medulla contained parts of different proximal and distal renal tubules. The outer layer of Bowman capsules was thin and lined by simple squamous epithelium. Bowman space was narrow, and the central glomerulus (capillary and its covering) showed normal high cellularity (Figure 7). The kidney tubules, both proximal and distal parts, showed normal epithelial lining and lumina. The nuclei of lining cells were rounded

and vesicular, and the blood capillaries surrounding the tubules were thin and mostly compressed (Figure 8).

Histology of supplemented rat kidney

In rat kidneys supplemented with the LD of vitamin (Figures 6-8), mild changes were observed with some renal corpuscles showing shrinkage and decreased cellularity of glomerular capillaries resulting in widening of Bowman space. A few tubules showed luminal dilation with deposition of hyaline material (casts). The ID (Figures 6-8) resulted in increased renal corpuscles with atrophy of glomerular capillaries and decreased cellularity. More tubules showed a decrease in epithelial height leading to luminal widening with the presence of some desquamated cells. The HD (Figure 6) resulted in focal areas of cellular damage. Most tubules showed dark cells with dark nuclei, which were the signs of cell apoptosis. In addition, necrosis, characterized by marked disorganization of renal tubules, hemorrhage and appearance of inflammatory cells, was seen in some samples, as shown in Figures 6-8.

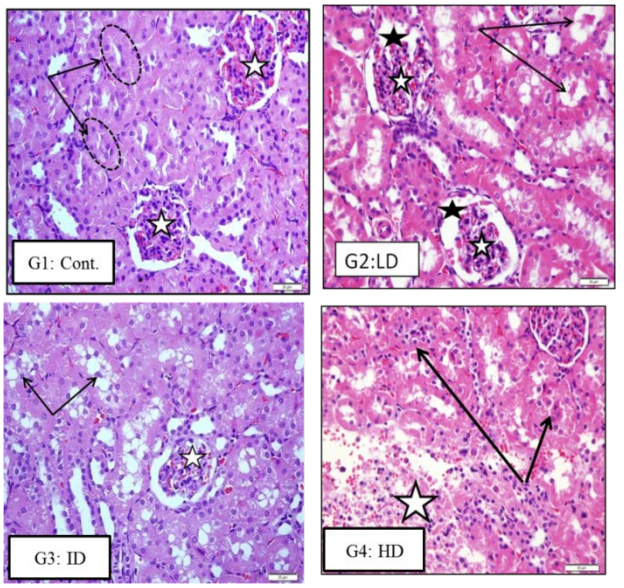

Fig. 6: Sections of rat kidney of different groups. (G1: Cont.) The control kidney showed normal renal corpuscle and glomeruli (white stars) and tubules (dotted circles and black arrows). (G2: LD) The LD kidney showed slight shrinkage of renal glomerulus (white stars) and wide Bowman space (black stars). Also shown, dilation of tubules with protein casts (black arrows). (G3: ID) The ID kidney showed the progression of glomerular atrophy (white stars) and cell apoptosis (black arrows). (G4: HD) The HD kidney showed marked necrosis (white star) and cell apoptosis (black arrows).

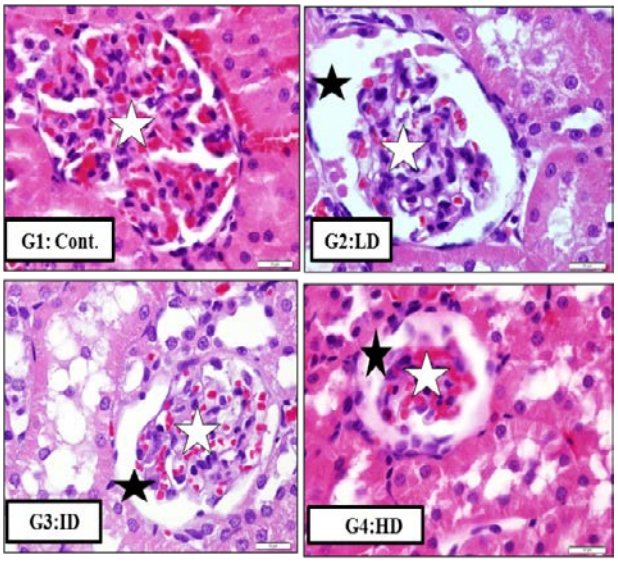

Fig. 7: Magnified sections of supplemented rats' kidneys compared to the control showing the changes in renal corpuscles with progressive atrophy of glomeruli (white stars) with degenerative changes (black star) in nearby tubules with increasing vitamin supplementation dose.

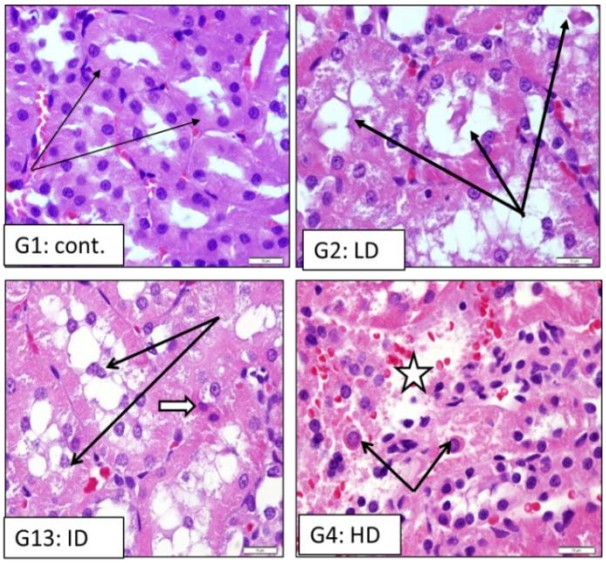

Fig. 8: Sections of rat renal tubules showing the effect of different doses of vitamin D on epithelial lining of the tubules. Note progressive dilation and increased hyaline casts in the LD (G2: LD) (arrows), epithelial desquamation (black arrows) and apoptosis (white arrows) in the ID (G3: ID) and necrosis and hemorrhage (white star) with plasma cell infiltration (black arrows) in the HD (G4: HD) group.

Histology of control rat heart

In sections taken from the left ventricle of the control heart, cardiac muscles were seen running in various directions. They were cylindrical in shape with ill-defined striations. They were branched fibers that were united

with each other. Their nuclei were oval, vesicular and central. Among the fibers, there was a scanty loose connective tissue with thin walled blood capillaries. The branches of coronary artery might be seen among the fibers. The artery was lined by simple squamous cells and had a thin muscular wall, as shown in Figures 9 and 10.

Histology of supplemented rat heart

With administration of the low vitamin D dose (Figures 9), the cardiac fibers looked healthier than in the control group. Slight changes and congestion were observed in the coronary arteries. In the ID heart (Figures 9), the enlargement of cardiac muscle was observed. Capillaries among the fibers were dilated and congested, while some samples showed dark degenerated fibers (apoptotic changes). In the HD heart (Figure 9), the local regions of damaged cardiac fibers were apparent, and inflammatory cells, including macrophages, were also seen. As the dose of vitamin D increased, there was an increase in wall thickness of the coronary arteries, shown in Figure 10, especially in rats receiving the ID and HD.

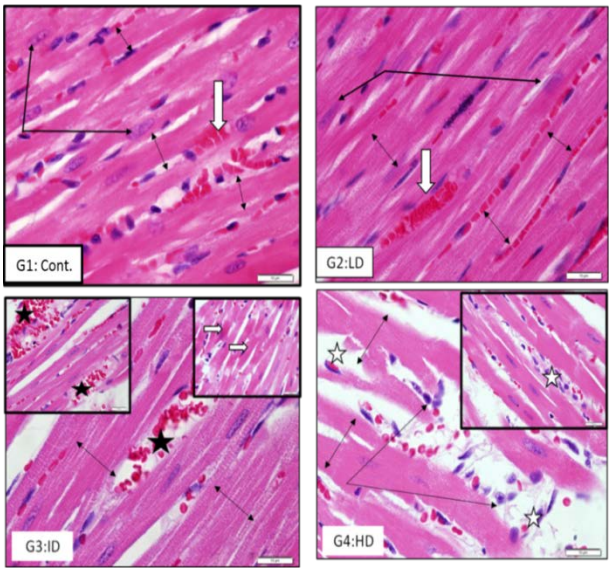

Fig. 9: Sections of rat cardiac muscle from the left ventricle showing: (G1: Cont.) fibers of normal thickness (double-headed arrows) having oval nuclei (thin black arrows). Blood capillaries are thin walled and non-congested (white arrow). LD sections (G2: LD) showing slight changes with slight fiber enlargement (double-headed arrows) observed in cardiac fibers. The ID sections (G3: ID) showed the enlargement of cardiac muscle (double-headed arrows) and dilated and congested capillaries (black stars and inserts). Some samples showed dark degenerated apoptotic fibers (white arrows and insert). The HD heart (G4: HD) showed local regions of cardiac fibers damage (white stars) with the presence of inflammatory cells and the infiltration of macrophages (thin black arrows).

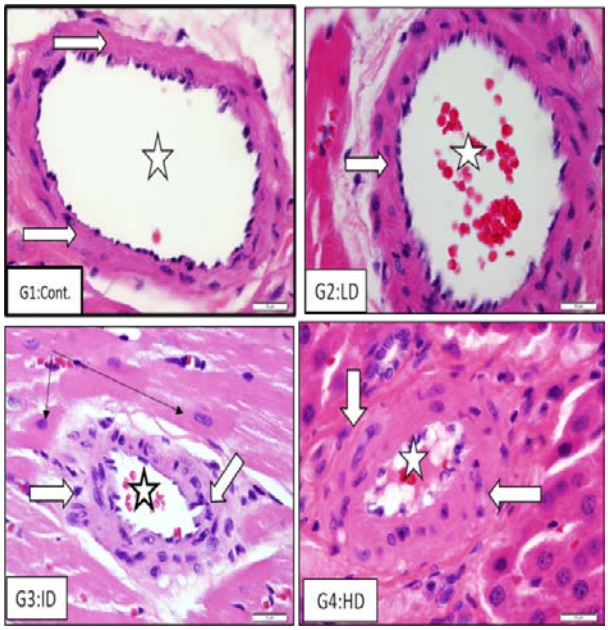

Fig. 10: Sections of the coronary artery from different rat groups. The control rats (G1: Cont.) showed normal wall thickness (white arrows) and wide lumen (white star). For the LD (G2: LD) the coronary artery still showed normal thickness. The ID (G3: ID) showed moderate thickening of the coronary wall with the proliferation of the muscular layer (white arrows) and narrowing of the lumen (white star). Nearby muscles showed dark staining (due to degeneration) (black arrows). Finally, the HD heart (G4: HD) showed marked thickening of the coronary wall (white arrows) and marked narrow lumen (white star).

DISCUSSION

This research study determined the effects of orally administered vitamin D, at three different doses, on weights and histological changes of organs involved in the immune system (spleen and thymus) and organs prone to calcification (liver, kidney, and heart) in male Wistar rats. The vitamin D levels used in this study are much higher than the levels considered toxic to rats by the other researchers [17-21] and thus these levels led to hypervitaminosis D. Many researchers have studied the effects of therapeutic, recommended the low levels of vitamin D on different aspects and parameters in humans and animals, but very few researchers have studied the effects of toxic or very high doses of vitamin D. In fact, there were no other research studies that used the very high doses used in the current study.

Signs of vitamin D toxicity were observed in the supplemented rats after the first week, such as neurological irritation, as evidenced by aimless running and rolling, dullness, weakness, rigidity of limbs and difficulty in movement and respiration. Shivering and

epistaxis were also observed before the death of two rats that received the high dose of vitamin D. Such symptoms of vitamin D toxicity were also mentioned in previous reports [15-17, 22].

The results showed no significant differences (P = 0.668, P = 0.337, respectively) between the groups for spleen and thymus mean weight indices. These results disagreed with previous studies [23] that found decreased thymus weights with high vitamin D levels. The results showed a significantly lower (P = 0.01) mean liver index for the ID group (mean ± SD: 3.46 ± 0.48) compared to the mean liver index for the control group (4.11 ± 0.53). In addition, the mean ID liver index was significantly higher (P = 0.000) and significantly lower (P = 0.019) compared to the mean indices for the LD (4.39 ± 0.40) and HD groups (4.07 ± 0.52); respectively. Other comparisons between the groups showed no significant differences. It has been well known that the liver is the major organ involved in vitamin D metabolism and it is one of the organs that may undergo calcification, as noted above. Thus, most other studies investigated the effects of vitamin D deficiency on the weights of organs and other parameters, but there have been very few studies that demonstrated the effect of vitamin D toxicity or overdose on liver weight. Chavhan *et al*. (2011) reported that there were no changes in liver weights in rats on a high dose of vitamin D [17].

The mean kidney index was highly and significantly lower (P = 0.003) for the ID group (0.74 ± 0.10) compared to the control mean kidney index (0.89 ± 0.12). In addition, the mean kidney indices for both the ID and HD groups (0.74 ± 0.10, and 0.80 ± 0.04, respectively) were, respectively, significantly higher and lower (P = 0.001, and P = 0.021, respectively) compared to the LD mean kidney index (0.92 ± 0.09). Other comparisons between the groups were not significantly different. The lower mean kidney index for the ID group might be due to the degenerative changes and parenchymatous necrosis observed in the histological examination. No previous studies showed the effect of hypervitaminosis D on kidney weight although, as mentioned above, the kidney is a major site for vitamin D metabolism and it is susceptible to calcification.

The mean heart index for the LD group (0.41 ± 0.04) was significantly higher (P = 0.022) than the mean heart index for the control group (0.37 ± 0.04). The mean heart indices for the ID (0.33 ± 0.03) and HD groups (0.35 ± 0.03) were both highly and significantly lower (P = 0.000 and P = 0.008 respectively) than the mean index for the LD group. Other within group comparisons were not significantly different. Thus, the mean heart index for rats given the LD was higher than the mean indices for rats of the control, ID, and HD groups. This might point to the improvement of structural integrity of cardiac muscle fibers, which was corroborated by the histological examination of the heart sections where muscle fibers looked more healthy and intact for the LD group compared to the control. In addition, the higher heart weight might be due to the hypertrophy of heart muscle or hyperplasia of muscle cells. There have been no other studies on the effects of hypervitaminosis D on the heart, making this study the first to show this. It is interesting to note that the mean liver, kidney, and heart indices for the ID and HD groups, with the exception of the mean liver index for the HD group, were all significantly lower than the respective mean indices for the LD group. Therefore, the ID and HD seemed to have resulted in toxic effects on the liver, kidney, and heart. On the other hand, the LD did not lead to any major or important changes.

The immune system in humans and animals includes both central (primary) and peripheral (secondary) immune organs plus circulating cells in the blood. The lymphoid organs are where lymphocytes, the most complex and important cells of the humoral (B cells) and cellular (T cells) acquired immunities and many of the functions of the immune system, originate, mature, proliferate, and become active against antigens. In the present study, the effects of different doses of vitamin D on immune system organs were studied with regard to changes in the thymus gland and spleen.

The thymus is a primary lymphoid organ, and it is where T lymphocytes become fully differentiated and mature. The thymus is made up of two lobes with a capsule surrounding each. Each lobe contains an outer compartment called the cortex that contains a high population of immature T cells, named thymocytes, which are the thymus cells that differentiate into mature T cells. The medulla, the inner part of the thymus, contains more mature thymocytes but at a less dense population. Other cells are present in the thymus such as dendritic cells and macrophages.

In the current study, histological examination of the thymus showed that the low dose of vitamin D did not affect the histological features of the thymus much. However, with increasing vitamin dose, depletion of the thymocyte cell population was observed in both the cortex and medulla. The thymus of rats receiving the high dose were highly affected compared to those receiving the low or intermediate doses. Reticulocytes with active large nuclei become more prominent especially within the medullary regions of lobules.

The spleen is the largest and one of the most important organs of the secondary lymphoid organs. The secondary lymphoid organs are where white blood cells come in contact with pathogens and, upon binding to these pathogens, become active and proliferate. In the spleen,

red cells are stored and old ones are destroyed. It purifies the blood and traps pathogens from the blood thereby initiating an acquired immune response against them. The spleen is contained within a capsule, which is a fibrous covering, and it is made up of red and white pulps. The red pulp is where macrophages, red blood cells, a few lymphocytes, and various blood components such as platelets reside and it is where old red blood cells are removed from circulation and destroyed. The red pulp is made up of a network of sinusoids, which are wide blood vessels, and cords of connective tissue. The white pulp contains mainly T lymphocytes, found in the periarterial lymphatic sheath (PALS) that surround the central artery, and also B lymphocytes in the lymph follicles, and macrophages and antigen presenting cells found in the germinal centers of the follicles. It is also where lymphocytes encounter antigens and become activated by them and lead to the acquired immune response.

In the present study, it was found that the administration of vitamin D in different doses resulted in histological changes in the spleen, such as focal thickening of splenic capsule, enlargement of the white pulp, and an increase in the area of active germinal centers. Those changes were dose dependent, and they were most evident in the spleen of the high dose group. In the HD group, the active germinal centers of splenic lymphatic nodules looked lighter with less lymphocyte population, and an increased number of macrophages containing cell debris. The study done by Stumpf *et al.* (1990) on hamsters injected with radioactively labeled vitamin D, revealed nuclear concentration of the vitamin in cells of the red pulp [24]. In the PALS, the labeled cells were found predominately at the outer rim, with a few scattered labeled cells in the inner PALS and in the marginal zone.

In the present study, it was observed that both thymus and spleen cells had lightly stained cytoplasm and contained cell debris (cell nests) that were dominant in samples receiving the high dose of vitamin D. These cells were similar to those described by Kojima *et al*. (1992) who reported that vitamin D_3 derivatives promoted the differentiation of monocytes into macrophages [25]. Rats receiving the high vitamin D dose showed the depletion of lymphocytes in the thymus. In addition, the high dose of vitamin D markedly increased the size of germinal centers and the appearance of macrophages containing cell debris.

In the liver, histological examination revealed scattered cells possessing highly acidophilic cytoplasm and dark nuclei. These changes were features of apoptotic cells. The accumulation of foci of inflammatory cells at sites of necrotic hepatocytes and around the bile duct was also observed. It was well known that 25-hydroxyvitamin D production from vitamin D occurred mainly in the liver through hepatic 25-hydroxlase enzyme activity, which was found in both mitochondrial and microsomal fractions [26]. Hydroxylated products were proven to possess the increased biological activity [27, 28]. This might occur in the high dose that induced parenchymatous degeneration such as what was seen in the present study. This could also affect the normal parenchymatous cells, including the liver, especially if vitamin D was given at higher doses and for a prolonged duration, as was the case here. In view of those reports, one can suggest that increasing the dose of vitamin D over therapeutic levels may have local toxic effects on the hepatocytes themselves.

In the kidneys, mild changes were observed in samples receiving the LD. Renal corpuscles showed shrinkage and decreased cellularity of glomerular capillaries, which resulted in widening of Bowman space. Few tubules showed luminal dilation with deposition of hyaline material (casts). In rats receiving the ID, more glomerular atrophy and decreased cellular density were observed. More tubules showed a decrease in epithelial height leading to luminal widening. Marked changes were observed in the kidneys of the HD, where focal areas of cellular damage were evident. Most tubules showed dark cells with dark nuclei, which were signs of cell apoptosis. In the rats taking the HD of vitamin D, necrosis, characterized by marked disorganization of renal tubules, hemorrhages and the appearance of inflammatory cells, was seen among the damaged tubules.

The deposition of calcium was not found in kidneys of the present samples even in rats receiving high doses. Chavhan *et al*. (2011) studied the effect of vitamin D_3 toxicity on the rat kidney [17]. They found that there was a deposition of calcium in the cortex and medulla, which was not found in the present study. However, coagulative necrosis and the presence of proteinaceous casts in the lumen were observed in kidneys of the ID and HD groups.

Vitamin D Deficiency was reported to enhance nephrotoxicity induced by many chronic diseases and oxidative chemical toxicity [29-32]. Some studies were found regarding the possible harmful effect of vitamin D overdose. Hypervitaminosis D was reported to result in excess calcium deposition in kidney parenchyma, which might result in the impairment of kidney functions [33, 34]. Conti *et al*. (2014) reported two cases of hypervitaminosis D in humans that resulted from self–administration of vitamin D and that led to signs of toxicity, including severe hypercalcaemia, nephrolithiasis and renal failure [35].

Degenerative changes, which were observed in the present study, with the absence of any features of calcium deposition, were found in the kidney parenchyma. This

may be due to the short period of the experiment or species variation in vitamin D metabolism. More specific investigations are needed to confirm the effect of hypervitamnosis D on kidney function and structures.

As for the heart in the present study, mild congestion of the coronary artery was observed with low vitamin D dose, while mild thickening of the arterial wall and mild degeneration in cardiac muscle fibers were observed in the ID. These changes were most evident with the administration of the HD where focal regions of cardiac muscle necrosis with hemorrhage were observed.

Other researchers studied the relationship between vitamin D and cardiac health and they pointed to the role of vitamin D deficiency as a risk factor for heart attacks, congestive heart failure, peripheral arterial disease (PAD), stroke [36-38]. Masterjohn (2007) found that hypervitaminosis D led to significant abnormalities in the heart and aorta, with the left side of the heart being more affected than the right [39]. The calcification of some valves along with white plaques on the luminal surface were also observed. No other literature on the direct effect of vitamin D overdose on heart histology were found. However, the increased calcium serum level that may result from high vitamin D doses might badly affect the cardiac function resulting in increased cardiac contraction, increased oxygen demand and consequent ischemic and degenerative changes [11]. In view of the previous data, the present degenerative and necrotic changes in the cardiac muscle of the HD group may be explained.

A previous study [40] reported that the removal of the VDR signaling system resulted in changes in the structure of the heart which pointed to a relationship between vitamin D and its effects on the heart, as the findings of this study also suggested. The histological examination [41] of the heart in vitamin D_3 deficient rats showed many changes in muscles and tissues of the heart.

Other than the few studies discussed above, no other studies on the effects of hypervitaminosis D on the histology of the immunological and vital organs studied here (kidney, liver and heart), or organ weights were found after the exhaustive searches in the literature available on the internet. Therefore, the findings of this study have been the first in this area using the high doses of vitamin D.

CONCLUSIONS AND RECOMMENDATIONS

In recent decades, vitamin D research has confirmed important interactions between vitamin D and the innate and adaptive immune systems. Natural as well as therapeutic doses of vitamin D were recommended to keep the integrity of most body functions including the immune system. The present study showed that vitamin D overdose might affect the general body activities. Clinical signs of neurological irritation, dullness, weakness, rigidity of limbs and difficulty in movement, respiration, shivering and epistaxis were observed in the rats and especially more so before death, probably, due to hypervitaminosis D.

In the present study, histological examination of stained sections showed that vitamin D in low doses did not alter the histological features of vital organs, namely the liver, kidney and spleen. Instead, the improvement of some parenchyma cellular features was observed. On the other hand, the ID produced minimum changes especially in kidney tubules. The high dose resulted in the focal degenerative changes in the studied organs.

Vitamin D doses had no significant effect on the spleen and thymus weights. The decrease in the kidney index with the increase in doses might be due to the degenerative changes and parenchymal necrosis, which were observed on histological examination. Statistical analysis pointed to the increase in the heart index of the LD group compared to the control, ID and HD groups. This might point to the improvement of structural integrity of cardiac muscle fibers, which was proved in this study by histological examination where muscle fibers looked more healthy and intact compared to the control.

Rats with the low dose of vitamin D showed normal cell population in both the thymus and liver. In addition, mild changes in renal corpuscles structure, enlargement of cardiac fibers and thickening of splenic capsule were found. Therefore, the therapeutic or low dose did not have significant effects. However, it significantly improved the studied parameters and histological structure of immune and vital organs.

Further future studies on the effect of vitamin D overdose on vital and immune system organs are recommended to further elucidate and confirm the changes and effects produced by hypervitaminosis D.

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