Journal of Applied Molecular Biology (JAMB)
MolecularBiology Research & Studies Institute, Assiut University, Egypt.
ISSN 2974-4008
Vol. 2(1). JAN. 2024
Journal of Applied Molecular Biology (ekb.eg)

Effect of Vitamin D Overdose on *VDR*, *TRPV6* and *CYP3A11* Genes Expression, Biochemical Tests and Histopathological Lesions in *Albino* Mice

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ARTICLE INFO

Article History:
Received:21-08-2023
Accepted:25-10-2023
Online: 16-01-2024

Keywords:
vitamin D, *VDR*, *TRPV6* and *CYP3A11* genes, histopathological lesions, biochemical effects.

ABSTRACT

Due to the spread of corona virus, many doctors' advice people to take vitamin D to prevent catching corona virus. But some people used it carelessly without keeping out the consequences of toxicity. For this reason, this study concerns on the impact of vitamin D overdose in mice on the expression of different important genes (*VDR*, *TRPV6* and *CYP3A11* genes), histopathological lesions, and biochemical properties. Forty male Albino mice, each male weighting 35-40 g, were randomly divided into four group each group contains 10 mice. Each group was treated daily with a different concentration of Vitamin D for 8 weeks by a stomach tube. The four groups were control (without Vitamin D), low dose (1um of Vitamin D /ml), intermediate dose (2 um of Vitamin D /ml) and high dose (3 um of Vitamin D /ml). After 8 weeks, the mice were slaughtered and different tissues were examined. Results showed that vitamin D overdose caused up regulation in the *VDR* gene expression, and down regulation in both *TRPV6* and *CYP3A11* genes. Moreover, vitamin D overdose caused significant histopathological changes that were observed in different tissues as stomach, heart, lung and kidney. Biochemical properties were investigated to test the liver function by measuring the ALT, and AST enzymes. While the Kidney function were examined by measuring BUN, vitamin D and calcium levels. The biochemical tests illustrated that vitamin D overdose caused raised level of total calcium, BUN, ALT and AST enzyme in blood. These signs referred to damage of kidney and liver relatively.

INTRODUCTION

Vitamin D is a group of fat-soluble seco-steroids responsible for many biological effects in human. The most important compounds in this group are D3 (cholecalciferol) and vitamin D2 (ergocalciferol) **(Bikle and Christakos, 2020).** The major natural source of vitamin D is synthesis of cholecalciferol in the lower layers of skin epidermis through chemical reaction that depend on sun exposure; specifically UVB radiation; 290–315nm **(Chow *et al.*, 2013)**. Cholecalciferol and ergocalciferol can be taken from the food and supplements. Only few foods, such as flesh of fatty fish. Mushrooms naturally contains significant amount of vitamin D. In the U.S and other countries, cow's milk and plant-derived milk substitutes are fortified with vitamin D as are many breakfast cereals **(Jäpelt and Jakobsen, 2013; Prietl *et al.*, 2013)**.

Vitamin D is responsible of many functions in human body as; Intestinal calcium and phosphate absorption **(Fukumoto, 2014; Alabada and Saleh, 2020)**, Calcium and phosphate reabsorption **(DeLuca, 2004)**, Bone remodelling **(Bikle, 2012)**, atherosclerosis **(C Brewer *et al.*, 2011; Kassi *et al.*, 2013)**, anticancer actions **(Picotto *et al.*, 2012)**, immune regulation **(Di Rosa *et al.*, 2011; L Bishop *et al.*, 2021)**, and xenobiotic detoxification **(Haussler *et al.*, 2013; Gil *et al.*, 2018)**.

Vitamin D deficiency are the result of many factors such as: Inadequate exposure to sunlight **(Holick, 2006)**, poor vitamin D diet **(Holick, 2006)**, gastrointestinal disorder **(Meeker *et al.*, 2016)**, renal diseases **(Holick, 2005; Dusso *et al.*, 2011)**, liver diseases **(Iruzubieta *et al.*, 2014)**, obesity **(Vanlint, 2013; Pereira-Santos *et al.*, 2015)**, malabsorption **(Margulies *et al.*, 2015)**, and genetic mutation in the forming enzymes such as; *CYP27A1*, *CYP2R1*, *CYP27B1***(Quach *et al.*, 2018)**.

Vitamin D deficiency can cause many issues such as; Mental disorder and depression **(Anglin *et al.*, 2013; Parker *et al.*, 2017)**, schizophrenia **(Berk *et al.*, 2007; Humble and Biology, 2010; McGrath *et al.*, 2010; Valipour *et al.*, 2014)**, tuberculosis infection **(Sasidharan *et al.*, 2002; Talat *et al.*, 2010)**, influenza infection **(Cannell *et al.*, 2006; Sundaram and Coleman, 2012)**, upper respiratory infection **(McNally *et al.*, 2009)**, Colon cancer **(Pereira *et al.*, 2012)**, prostate cancer **(Schwartz and Hulka, 1990; Tuohimaa *et al.*, 2001)**, breast cancer **(Lowe *et al.*, 2005; Kim and Je, 2014)**, pancreas cancer **(Norman *et al.*, 1980; Hoogenboom *et al.*, 2016)**.

Vitamin D from the diet or from skin synthesis, is initially produced in a biologically inactive form which is useless to the body and needs an activation. this activation occurs by two different genes *(CYP2R1* and *CYP27B1*). *CYP2R1* gene convert vitamin D3 to 25 (OH)D3, while *CYP27B1* gene convert 25(OH) D3 to the active form 1.25di(OH)D3 (Berridge, 2017; Charoenngam and Holick, 2020).

Binding vitamin D to VDR (vitamin D receptor) plays an important role in preserving the concentration of calcium, phosphate, parathyroid hormone (PTH) in the blood **(Haussler *et al.*, 2013; Goltzman *et al.*, 2018)**. Moreover, it controls the level of activated Vitamin D in vital organs as kidney, liver, intestine and parathyroid gland **(Henry and Norman, 1984; Lorè *et al.*, 1987)**. In addition, the vitamin D –VDR complex could prevent the hypertrophic activity in cardiac myocytes when it is given in normal doses **(Gardner *et al.*, 2013)**.

The VDR mainly located in the cytosol and after binding to its ligand (vitamin D), it is rapidly translocated to the nucleus where the complex functions as a trans activating transcription factor **(Ryan *et al.,* 2015)**.

The binding of vitamin D with the VDR controls the levels of vitamin D by decreasing *CYP27B1* gene expression (in low levels of vitamin D) **(Murayama *et al.,* 1999)**, and stimulating the expression of the degradation enzyme (*CYP24A1* gene) (in high levels of vitamin D) **(Meyer *et al.,* 2007)**.

In the intestine and kidney, vitamin D maintain calcium and phosphate levels in plasma via the transient receptor potential cation calcium channels (*TRPV6*) and sodium-coupled phosphate cotransporter (NaPi) **(den Dekker *et al.,* 2003; Takeda *et al.,* 2004; Meyer *et al.,* 2006)**. So that *TRPV6* gene and *VDR* gene are mainly responsible of stability of normal concentration of calcium and phosphate in blood **(Christakos *et al.,* 2016)**.

RXR (retinoid X Receptor) can react with vitamin D - VDR complex to create a colon anticancer substance **(Han *et al.,* 2010; Haussler *et al.,* 2013)**. This complex activates (upregulation) *CYP3A11* gene in colon of to convert LCA (lithocholic acid), a toxic secondary bile acid generated in the enteric system by bacteria, to 6aOH LCA which reacts with 3aSo4LCA to form colon anticancer substance. In mice *CYP3A11* gene is equal *CYP3A4* gene in human **(Haussler *et al.,* 2013)**. Human CYP3A4 (Cyp3a11 in mice) plays an important role in forming enzymes of drug metabolism and detoxification **(Tong *et al.,* 2019)**.

Vitamin D play an important role in curing Covid-19 symptoms, which infect the upper respiratory system **(Devaux *et al.,* 2020)**, since it can activate the immune system against different infectious pathogens. So, many doctors recommend people to take vitamin D during the epidemic of corona virus **(Mansur *et al.,* 2020)** but some patients used it unwisely without taking into account the consequences of the vitamin D overdose effect on their health.

There are many studies concerns on vitamin D deficiency, Vitamin D deficiency led to many issues for the body. On other hand, the vitamin D overdose was rarely investigated compared to vitamin D deficiency studies.

Previous study showed that mice taking vitamin D with overdoses suffer from hypercalcaemia **(Cui *et al.,* 2012)** due to an activation of TRPV6 channel, responsible of absorption of calcium. Moreover, it caused calcification in the wall of stomach and aorta **(Chavhan *et al.,* 2011)**. Toxicity doses of vitamin D resulted in the separation of muscle fibres, muscle cell degeneration, and glomrulitis **(Singla *et al.,* 2015)**.

Due to the insufficient investigation on the effect of vitamin D overdose, this study aimed to evaluate the impact of Vitamin D overdose on mice. Different effects were studied including the effect on the expression of different important genes (*VDR, TRPV6* and *CYP3A11*), liver function, Kidney function, cardiovascular function, and histopathological lesions.

MATERIALS AND METHODS

2.1. Experimental Animal

40 Male mice were used in this experiment; every ten mice were housed in a cage with a 12-h light/12-h dark cycle in a room at a controlled temperature (22 ± 2 °C) for 8 weeks. Mice received a semi-synthetic basal diet which is consists of: Starch 388, Casein 200, Sucrose 200 Soya oil 100 Vitamin-mineral mixture 60, Cellulose 50, DL-methionine 2, Calcium 5, and Phosphorus 3 g kg^{-1} **(Kotwan *et al.*, 2021)**.

The diets were kept refrigerated until they were fed to the mice. Food and water were provided twice daily. The 40 mice were randomly divided into 4 equal groups (10 mice each) **(Nadimi *et al.*, 2020)** as following:

Group 1: Control mice weren't supplied with any external vitamin D.

Group 2: low dose group, every mouse was administrated daily with 1ml of corn oil containing 1um of vitamin D.

Group 3: intermediate dose group, every mouse was administrated with 1ml of corn oil which contains 2 um of vitamin D.

Group 4: high dose group, every mouse was administrated with 1ml of corn oil containing 3 um of vitamin D.

After 8 weeks, the mice were slaughter and different tissues were examined**.** Intestine is extracted from each mouse to do RT-PCR test. Kidney, heart, lung and stomach are collected for histopathological examination. Blood is collected in tubes to perform the biochemical tests including measurement level of vitamin D, calcium, blood nitrogen urea, ALT and AST enzyme.

2.2. Vitamin D Overdose Effect on Gene Expression.

qRT-PCR experiment was performed to quantify the expression fold change of some genes. The expression of several genes was studied including: *VDR* and *TRPV6* due to their role in calcium absorption. The *CYP3A11* was studied because its role in forming anticancer substance in the intestine.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene in this study. It plays an important role in energy metabolism and the production of ATP. Recently (GAPDH) gene is used as "housekeeping" gene in gene expression studies as it become constant in cells or tissue under investigation **(Barber *et al.*, 2005)**.

The intestine from each mouse in the group was mixed and the mix was used to isolate total RNA. The intestine was used to isolate RNA because the absorption of calcium and anticancer formation occur in that tissue.

Total RNA was extracted using Abt total RNA mini extraction kit by following the manufacturer's instructions. RNA concentration and purity were measured in Nano Drop Spectrophotometer ND1000 (Nano Drop Technologies). The highly purified RNA was stored at -80 ºC for long term storage or at -20 ºC for short term storage use. After that, all RNA samples were diluted to a final concentration of 50 ng/µL and a reverse transcription for the RNA samples were performed with the first-strand cDNA Synthesis Kit (COSMO) using Poly A primers.

The q RT-PCR was performed at the Molecular Biology Research Centre (MBRU), Asyut University in a Bio-Rad iCycler. The q RT-PCR master mix for each gene was prepared by the following reaction components: 12.5µl iQ SYBR Green Super Mix (Invitrogen), 0.25µl of each primer (10µM) (table 1), and 8µl nuclease-free water. Then,

4µl of the cDNA were added to a 21µl of the master mix. The following PCR protocol was used: initial denaturation (95°C for 10 minutes), followed by repeated 40 cycles (denaturation: 95°C for 15 sec., annealing: 60°C for 20 sec., 72°C for 60 sec. with a single fluorescence measurement), followed by 72°C for 7 min and then cooling to 4°C.

For quantification of gene expression, the cycle threshold (Ct) was determined for each gene transcript. The Relative Quantification (ΔΔCT) method was utilized to calculate fold change **(Livak and Schmittgen, 2001; Rao *et al.*, 2013)**. The gene expression of all tested genes was normalized with the absolute expression mean values of the reference gene (*GAPDH*) **(Dheda *et al.*, 2005; Tong *et al.*, 2009; Brattelid *et al.*, 2010)**.

2.3. Hypertrophy of Heart.

Several studies showed that vitamin D deficiency stimulated the renin-angiotensin-aldosterone system and could do hypertension and left ventricular hypertrophy **(Achinger and Ayus, 2005; Lee *et al.*, 2008; Gupta *et al.*, 2012)**. In this study, the heart of each mouse in every group (n=10) was weighed using sensitive balance, the hearts were preserved by formalin to study the effect of vitamin D overdose on the heart weight.

2.4. Histopathology Studies.

Samples were collected from all groups (control and treated groups) after anesthetizing with chloroform then scarification of mice. The tissue specimens were taken from kidney, lung, stomach and heart. Samples from previous organs were cut in small pieces then washed by normal saline and fixed in 10% neutral buffered formalin for 24-48 hrs. After that, they were washed and dehydrated in graded alcohols. Paraffin blocks were prepared and step sections were cut at 5-7 microns' thickness. Sections were stained with haematoxylin and eosin stain (H&E stain) for routine histopathology.

2.5. Biochemical Studies.

The blood serum was collected by centrifugation of whole blood in wiserman tube at 1500 rpm for 15 minutes. This serum is used for the estimation of different biochemical measurements including (level of vitamin D, calcium, blood nitrogen urea, ALT and AST enzyme). The biochemical tests were estimated on auto analyser by using diagnostic reagent kits supplied by Bayer Diagnostics India **(Chavhan *et al.*, 2011)**.

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RESULTS

3.1. Mortality of Mice in Groups

In vitamin D high and intermediate doses groups, two mice were died before the end of the 8 weeks. The Died mice showed irregular precipitation of calcium in bones especially in the backbone **Figure (1)**. Similar results were obtained by **Williamson *et al.*, (2017)** and Kocher *et al.*, (2010) who domenstrated that Vitamin D3 supplementation

of lethal dose causes tougher bone that is more flexible and less fragile than that of mice supplied with standard levels of dietary vitamin D3.

3.2. Vitamin D Overdose Effect on *VDR, TRPV6* and *CYP3A11* Gene Expression.

In intestine tissue, the impact of vitamin D overdose on changing the gene expression levels for *VDR, TRPV6* and *CYP3A11* genes were investigated **Figure (2**).

The results showed that by increasing the vitamin D dose, it caused upregulation in *VDR* gene expression and downregulation of *TRPV6* and *CYP3A11* gene expression.
VDR gene is responsible of the activation of calcium channel in intestine (Cui *et al.*, 2012). Increasing in *VDR* gene expression leads to more uptake of calcium which causes hypercalcaemia. And probably this hypercalcaemia explains the reasons of killing mice in the overdose vitamin D groups. This result agrees with (Kocher *et al.*, 2010; Williamson *et al.*, 2017) who illustrated that Vitamin D3 supplementation of lethal dose of vitamin D killed all rats in their expermints.

Figure (2) showed that by increasing the vitamin D dose, it caused the downregulation in *TRPV6* gene expression. *TRPV6* gene is responsible of forming the transport calcium channel from intestine to blood (Cui *et al.*, 2012). As a result of Vitamin D overdose, the calcium concentration in blood was increased extremely and the body will try to overcome this problem by reducing calcium absorption and lowering the expression of *TRPV6* gene. This result contradict with the results obtained by **Cui *et al.*, (2012)** when mice treated with overdose vitamin D, *TRPV6* gene expression was upregulated.

Results in **Figure (2)** demonstrated that *CYP3A11* gene expression was downregulated as a consequence of increasing in vitamin D dose. *CYP3A11* gene plays an important role in forming anticancer colon substance **(Han *et al.*, 2010; Haussler *et al.*, 2013)**. The downregulation in this gene means an increasing probability of suffering of colon cancer diseases. **Wang *et al.* (2008)** proved that vitamin D stimulated *CYP3A11* mRNA levels in mice hepatocytes. Moreover, several research showed that vitamin D induced *CYP3A* gene, a human similar gene to *CYP3A11* gene **(Haussler *et al.*, 2013)**, expression in human both intestine and liver cells **(Wang *et al.*, 2008; Qin and Wang, 2019)**. These results were disagreement and opposite with the results obtained by **(Wang *et al.*, 2008; Qin and Wang, 2019)**

3.3. Weigh of Heart of Mice in Different Groups

Figure (3) and **table (2)** showed that by increasing the level of vitamin D, the weight of heart was increased regularly. Vitamin D deficiency stimulated the renin-angiotensin-aldosterone system and could do hypertension and left ventricular hypertrophy **(Lee *et al.*, 2008)**. These findings mean that the change of vitamin D treatment whether by increment or decrement will lead to the hypertrophy of heart.

3.4. Histological Changes in Different Tissues Caused by Vitamin D Overdose.
3.4.1. In Kidney:

The nephron consists of glomerular capsule (Bowman's capsule), proximal and distal convoluted tubules and nephron loop. The duct system consists of arched and straight collecting tubules, and papillary ducts **Figure (4a), & Figure (6a).**

Histopathological finding: In this study the kidney of treated mice of different groups showed partial (in group low and intermediate dose) to complete (in group high dose) obliteration of Bowman's space with congestion of mesangial blood vessels and expanded mesangial matrix **Figure (4)**. This is consistent with the results of **(Singla *et al.*, 2015)** with addition of presence of glomerulitis leading to decrease in Bowman's capsule space in treated rats. **(White *et al.*, 1984)** observed shrinkage in some urinary glomeruli as a result to the toxicity of the vitamin D with the expansion of capillary blood vessels. Occasionally, observed focal calcification in the renal tissue after long exposure to vitamin D toxicity as in group of high dose treated mice represented in basophilic clumps **Figure (5)**. In case of vitamin D3 toxicity in sheep, the medulla region of collecting tubules observed to be the major mineralisation site **(Simesen *et al.*, 1978)**. Mineralization (nephrocalcinosis) was noticed in cortex and medulla **(Chavhan *et al.*, 2011)**. In this study marked congestion along with vascular degeneration of varying severity in the epithelium of renal tubules and haemorrhage in intestitium was also observed. Swelling of the urinary epithelium may be due to hydropic degeneration **(Kumar *et al.*, 2017)**. Hyaline casts observed in renal tubular lumen with sever inter tubular haemorrhage in all treated groups **Figure (6)**. These was as a result of damage to the kidney tissue, and this agree with (Chavhan *et al.*, 2011) who reported that the tubular epithelium showed calcification and coagulative necrosis along with presence of proteinaceous casts in the lumen. In this study was reported diffuse cellular infiltration of varying severity in the renal tissue of all treated mice.

3.4.2. In Lung:

The alveoli are the structural and functional units of the respiratory system **Figure (7a) & Figure (8a)**.

Histopathological finding: In lung of treated groups, oedema and emphysema was noticed in all treated groups. Peri bronchial cellular infiltration sever in group (high dose) and mild in other treated groups (low and intermediate dose). Congestion of peri bronchial blood vessels and inter alveolar blood vessels **Figure (7). (Kocher *et al.*, 2010)** exhibited that the lung parenchyma had varying degree of deposition ranging from mild to diffuse deposits with clear congestion of alveolar capillaries and haemorrhages in the air spaces. **(Chavhan *et al.*, 2011)** also agree with our results. He reported brown dirty colour of granular deposits of calcium in alveoli and inter alveolar septae with cellular infiltration and thickened alveolar septae. Haemorrhage, emphysema, and edema were also reported. In this study, thickening of alveolar wall in all treated groups with cellular infiltration were observed. **(Chineme *et al.*, 1976)** explained that the deposition of calcium occurs in alveolar septa, bronchial sub-mucosa and wall of arteries in the pigs having vitamin D toxicity. In this study we observed that in the lung of group (intermediate and high dose), brown deposits intra- and inter alveolar represented by hemosiderin pigmentation with sever interalveolar haemorrhage. There was cellular infiltration in the thickened alveolar septae **Figure (8).**

3.4.3. In Stomach:

Histological finding: The stomach of mice is divided into two portions, non-glandular and glandular portion. Each portion consists of mucosa, submucosa, muscle

layer and serosa. The mucosa is subdivided into three layers, epithelial layer, lamina propria and muscularis mucosae. non-keratinized while in glandular portion columnar epithelium **Figure (9 a & b)**.

Histopathological finding: The stomach of treated mice had sloughing of inner gastric wall and mineralized. Basophilic clumps were observed in mucosa, muscularis mucosa and muscularis externa layers of stomach. Massive cellular infiltration in different layers of stomach especially in group (high dose) and mild in group (low and intermediate dose). This is an agree with **(Kocher *et al.*, 2010)** who observed a mild lymph mononuclear cell infiltration, at some locations, in the sub epithelial layer of the stomach Haemorrhagic gastritis was also observed in some mice. Clear congestion of stomach blood vessels will be observed **Figure (9).** This agreement with the results of **(Chavhan *et al.*, 2011)**.

3.4.4. In Heart:

Histological finding: The heart is divided into the following layers: epicardium, myocardium, and endocardium. A double-layer, filled sac called the pericardium surrounds the heart. The pericardium is composed of the outer parietal and the inner visceral pericardium. The epicardium constitutes the visceral pericardium with fibro-elastic connective tissue, and adipose tissue. Coronary arteries and veins, lymphatic vessels and nerves are present below the epicardium. The endocardium consists of the endothelium and the sub endothelial connective tissue layer. The sub endocardium is present between the endocardium and myocardium and considered the impulse-conducting system.

Histopathological finding: Histopathological study of heart of treated mice revealed varying stages of mild to severe vascular degeneration of the heart muscles. Calcification was observed in the cardiac muscle fibres of group (high dose) treated mice. This is consistent with the results reported by **(Singla *et al.*, 2015)** who was reported that the toxicity of vitamin D leading to cardiac muscle separation, degeneration and necrosis of muscle cells in heart of treated rats. Next to these changes, there was mild focal infiltration of lymph mononuclear cells in the myocardium **Figure (10)**. Calcification was observed in epicardium, myocardium endocardium and cardiac valves. Myocardial haemorrhage with cellular infiltration and congestion of cardiac blood vessel were observed in all treated groups. Degeneration and replacement of cardiac muscle cells with fibrous tissue and cellular infiltration were observed by **(Chavhan *et al.*, 2011)**. Also he reported that the epicardium and myocardium were severely affected with extensive fibrous tissue proliferation.

3.5. Biochemical Analysis Results

Figure (11) illustrated that by rising the vitamin D dose treatment in different groups, there were a gradually increment of the vitamin D level in blood.

Vitamin D overdose leads to rise blood calcium level **(Figure 11)**. Higher level of blood calcium is due to the hyper excess of calcium absorption from intestine, decreasing calcium excretion by kidney, and by increasing mobilization of calcium from bone (Chavhan *et al.*, 2011; Anderson *et al.*, 2018).

Figure (11) also showed that by rising up the vitamin D dose treatment in different groups, there were a gradually increment in blood nitrogen urea levels. This could be due

the Hypercalcemia which leaded to an irreversible calcium precipitation in the kidney cortex and medulla. This precipitation causes a partially kidney damage **Figure (5)** leading to the increment of urea level in blood.

On the other hand, **Figure (12)** showed that by increasing vitamin D levels, higher levels of liver enzymes (ALT and AST) were obtained. This may be a sign of liver damages caused by vitamin D overdose. This result was also obtained by **Singla *et al.* (2015)** who treated rats with overdose vitamin D **(Singla *et al.*, 2015)**. The rats suffered from extreme weight loss, badly respiration, difficultly in movement and haemorrhage in some organs. These signs indicated that the vital organs of rats were severely damaged especially liver. The damage of liver occurred because of degeneration of cell and severally haemorrhage in cells. This damage in liver cells leaded to increase in its enzymes ALT and AST in blood.

CONCLUSION

The experiments focus on impact of different overdose of vitamin D concentration on several features in mice. The results illustrated that overdose of vitamin D caused changes in some gene expressions. *VDR* gene was upregulated, while both *TRPV6* and *CYP3A11* genes were downregulated. In addition, vitamin D overdose affected badly many tissues as kidney, lung, heart, and stomach. These tissues had dangerous lesions and damaged gradually according to the rise of dose. In biomedical tests, overdose of vitamin D leaded to rise levels of total calcium, urea, ALT and AST liver enzyme in the blood. Increment of urea levels in blood indicated to partial or total kidney damage. While higher levels of ALT and AST are signs of a dysfunctional liver. According to these findings, vitamin D must be taken only under medical supervision.

5. Ethical Approval

The study protocol was approved by the MBRSI-Research Ethics Committee, Asyut University, Egypt with the approval number: IORG0010947- AB-21-30-A.

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Tables: -

Table (1): primes used in the q RT-PCR

Primer	**Forward (5′→3′ sequence)**	**Reverse (5′→3′ sequence)**
VDR	GAGGTGTCTGAAGCCTGGAG	ACCTGCTTTCCTGGGTAGGT
CYP3A11	TCACACACACAGTTGTAGGGAGAA	GTCCATCCCTGCTTGTTTGTC
TRPV6	CTGCAAAGGGTTAGGGAGGC	TACAGGCACCAGTCTAGGGG
GAPDH	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG

Table 2: Means of heart weight in different groups

.

	Mean (mg)	**LSD***
Control	198.9	a
Low	210.0	b
Intermediate	217.4	b
High	231.8	c

*Values in the column followed by different letters indicate significant differences among treatments according to LSD test at 0.05.

Figures: -

Figure (1). Died mice in vitamin D overdose group, showed irregular precipitation of calcium in the backbone.

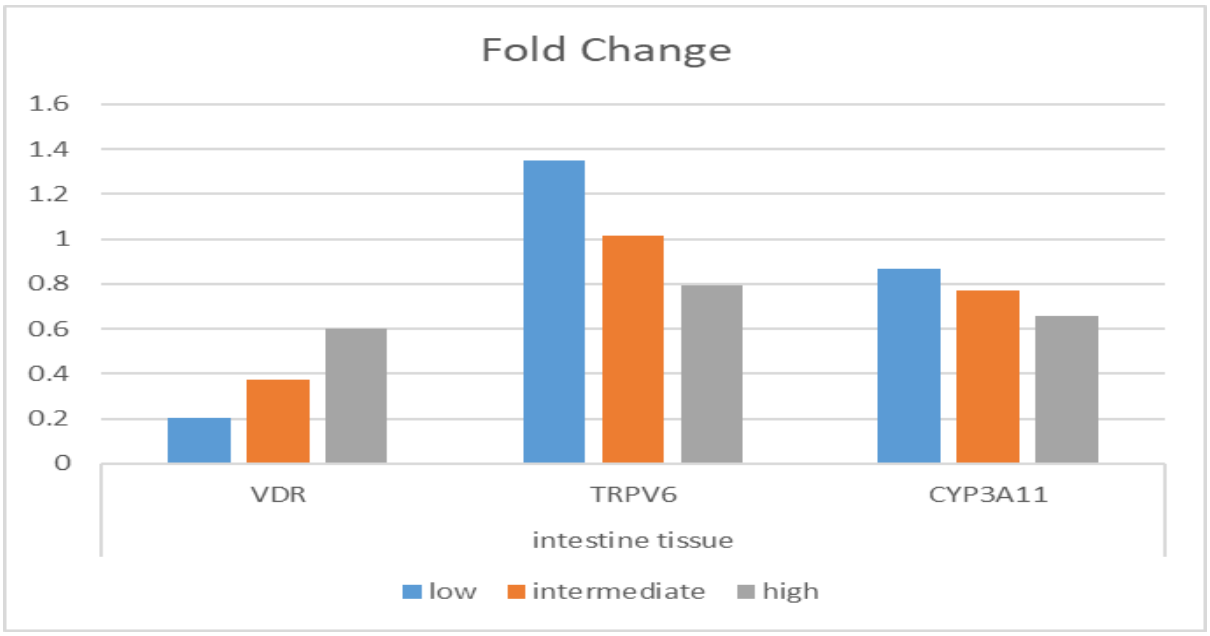

Figure (2). Fold change of *VDR*, *TRPV6* and *CYP3A11* genes caused by different vitamin D doses.

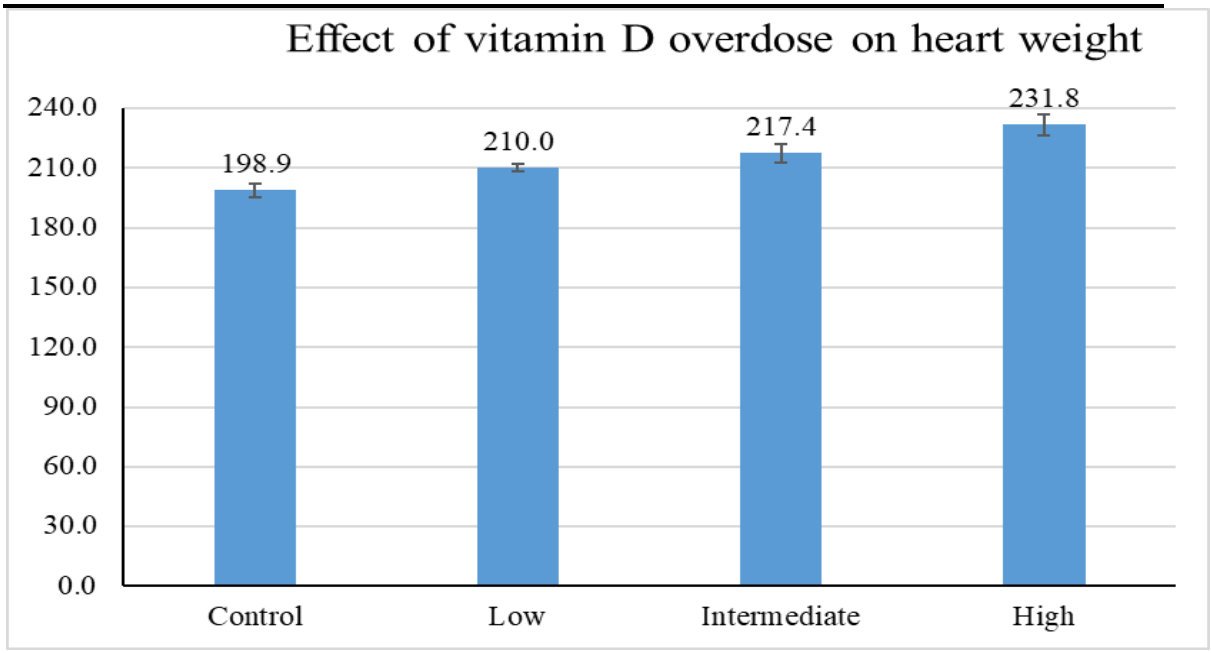

Figure (3). The impact of vitamin D overdose on heart weight

Results are mean ± SD.

Results were compared by LSD test.

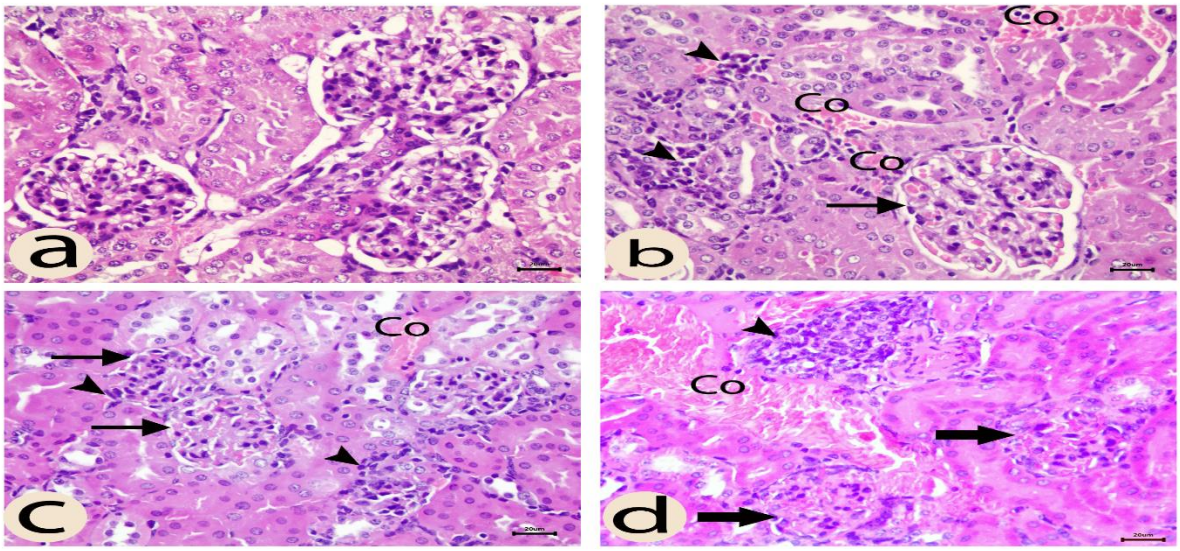

Figure (4). Section of kidney in different groups; (a) control group, (b) low vitamin D dose group, (c) intermediate vitamin D dose group, and (d) high vitamin D dose group.
a. control group with Bowman's capsule (normal).
b. c. partial obliteration of Bowman's space with congestion mesangial blood vessel, (thin arrows).
d. showing complete obliteration of Bowman's space with expanded mesangial matrix (black arrows).
Note (co) (congestion of inter tubular blood vessel) Arrow heads (cellular infiltration) H & E stain * 40.

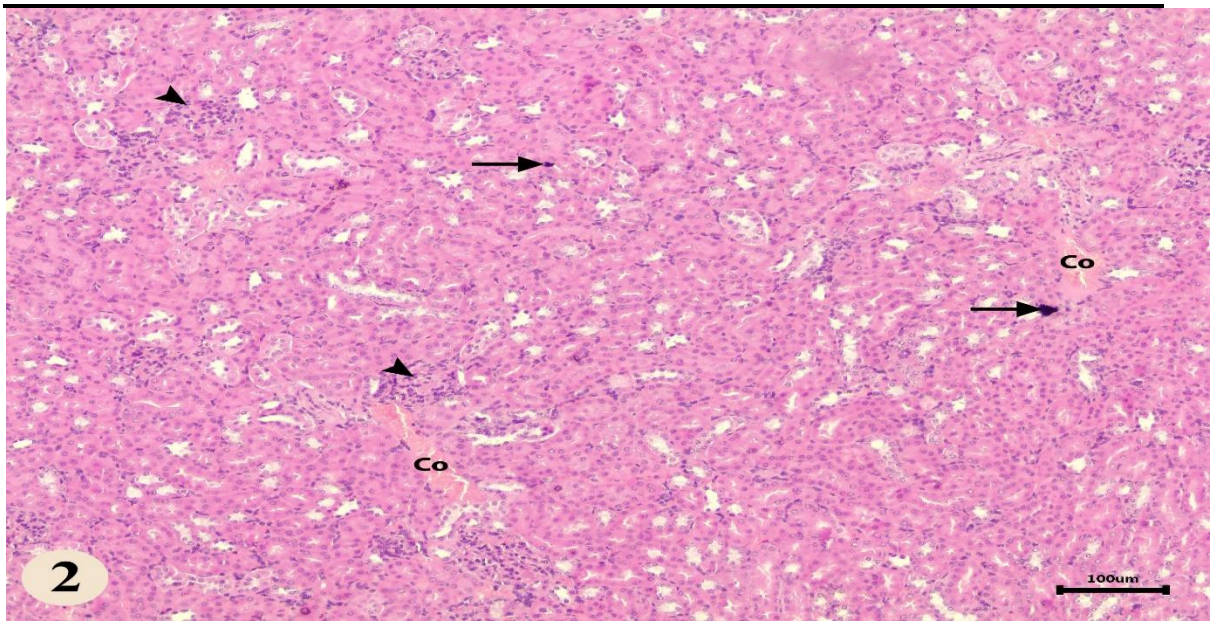

Figure (5). Section of kidney of high dose group.
A. basophilic clumps occasionally (arrow).
B. congestion (co).
C. cellular infiltration (arrow heads) H & E stain * 10.

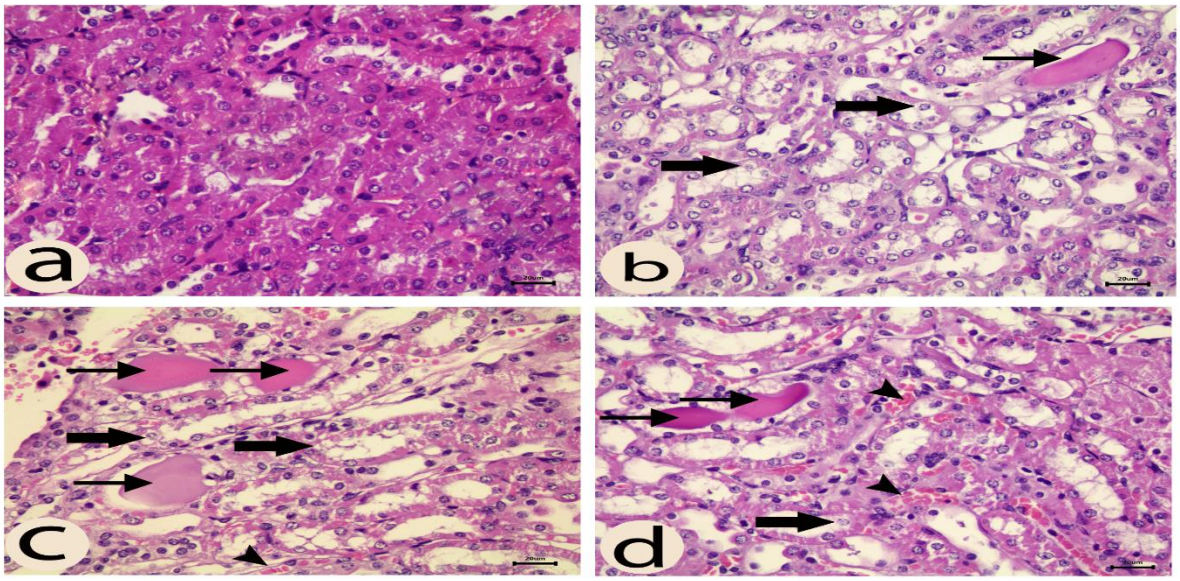

Figure (6). Section of renal tubules in different groups; (a) control group, (b) low vitamin D dose group, (c) intermediate vitamin D dose group, and (d) high vitamin D dose group. Hyaline in renal tubular lumen in all treated groups (thin arrows) with vascular degeneration (thick arrows) sever inter tubular haemorrhage in group (arrows head) H & E stain * 40.

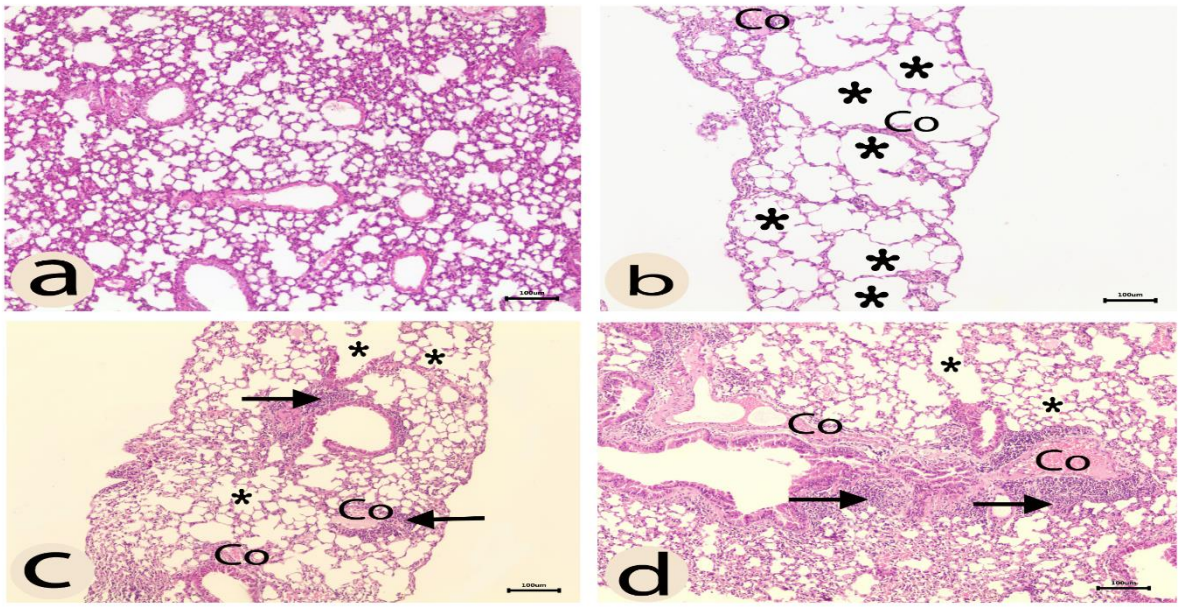

Figure (7). Section of lung in different groups; (a) control group, (b) low vitamin D dose group, (c) intermediate vitamin D dose group, and (d) high vitamin D dose group. Emphysema of lung tissue (star) note, peri bronchial cellular infiltration (arrows), sever Congestion of peri bronchial blood vessel and inter alveolar blood vessel (co) H & E * 10.

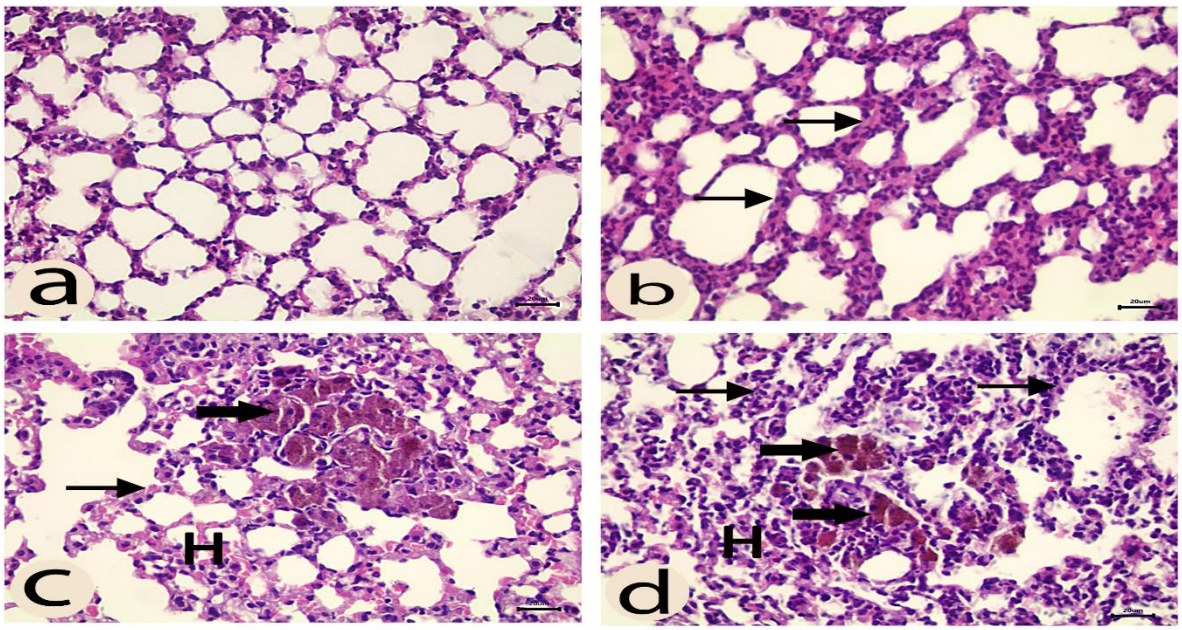

Figure (8). Section of lung of control group is (a), low dose group is (b), intermediate dose group is (c) and high dose group is (d). (c, d) hemosiderin pigmentation intra and inter alveolar (thick arrows). Thickening of alveolar wall in all treated groups (thin arrows) with cellular infiltration inter alveolar haemorrhage (c, d) (H). H&E *40.

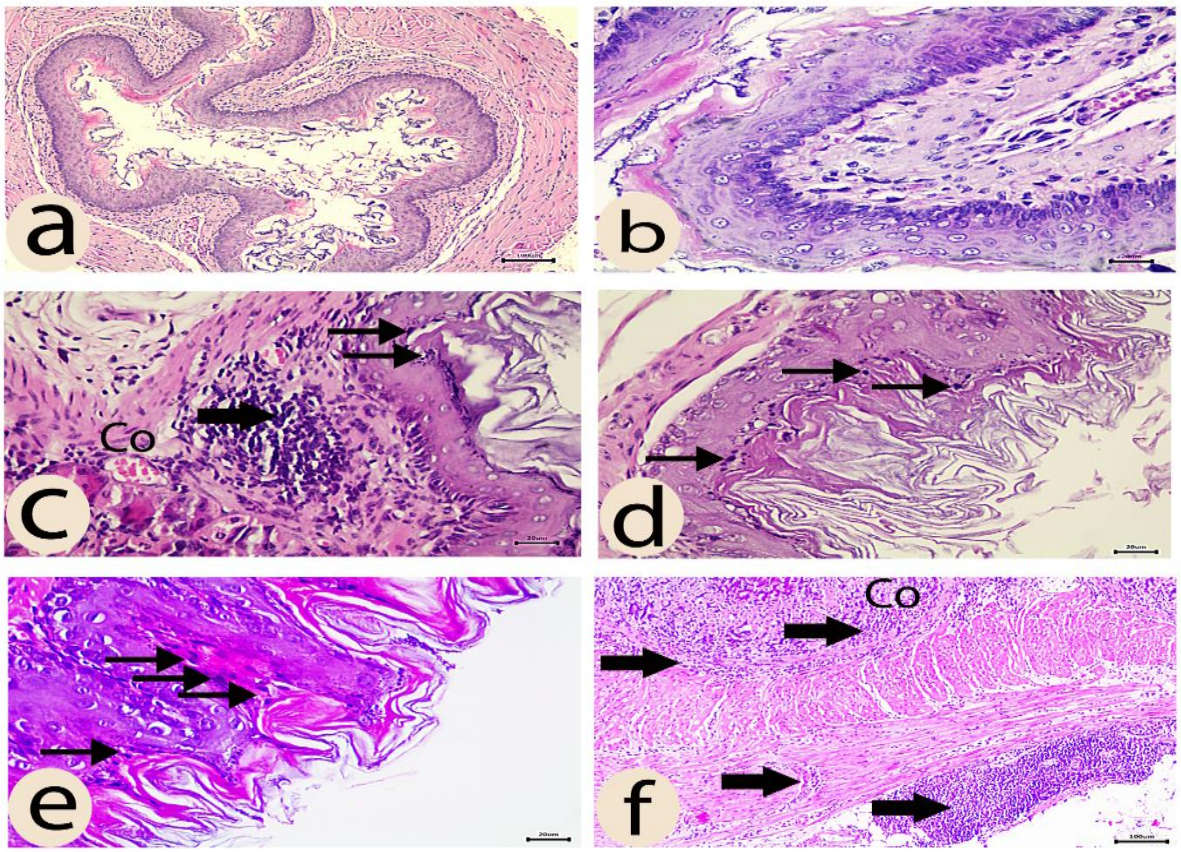

Figure (9). Section of stomach in control group is (a, b), low dose group is (c, d), intermediate dose group is (e) and high dose group is (f). Basophilic clumps in the epithelia layer of non-glandular part of mice stomach in all groups (thin arrows) with massive cellular infiltration in different layers of stomach (thick arrows). Congestion of stomach blood vessel (co) H&E stain *10 *40.

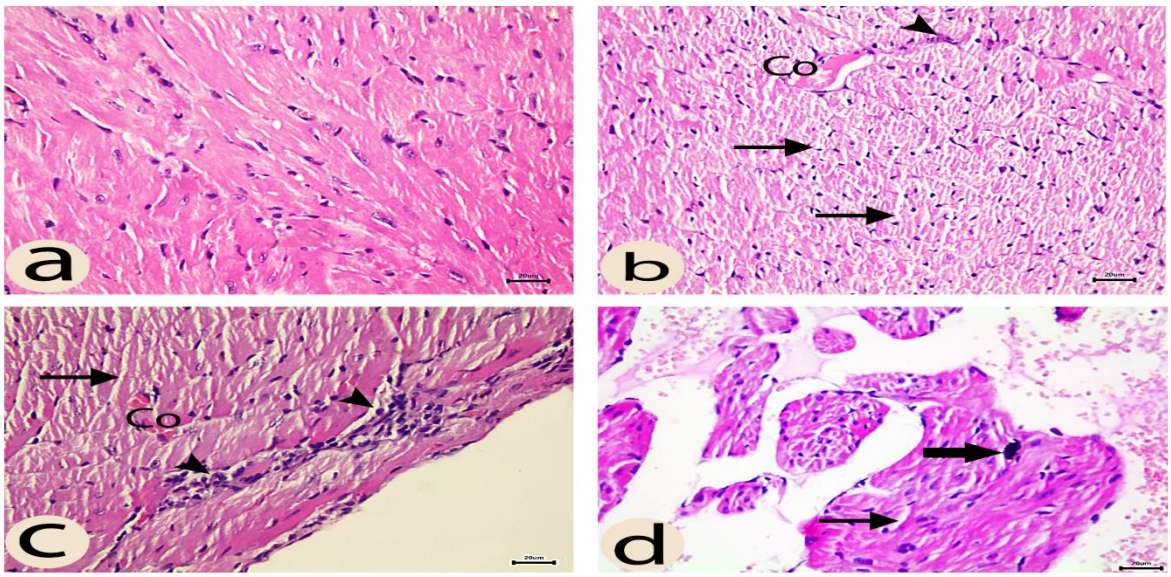

Figure (10). Section of heart in different groups; (a) control group, (b) low vitamin D dose group, (c) intermediate vitamin D dose group, and (d) high vitamin D dose group. Basophilic clumps in group 1 treated mice heart (thick arrow), a, vacculation and degeneration of myoepithelia cells in all treated groups (b, c, d) (thin arrows). haemorrhage between. Myoepithelial cells with cellular infiltration (arrow heads), congestion of cardiac blood vessel (co) H & E stain * 40.

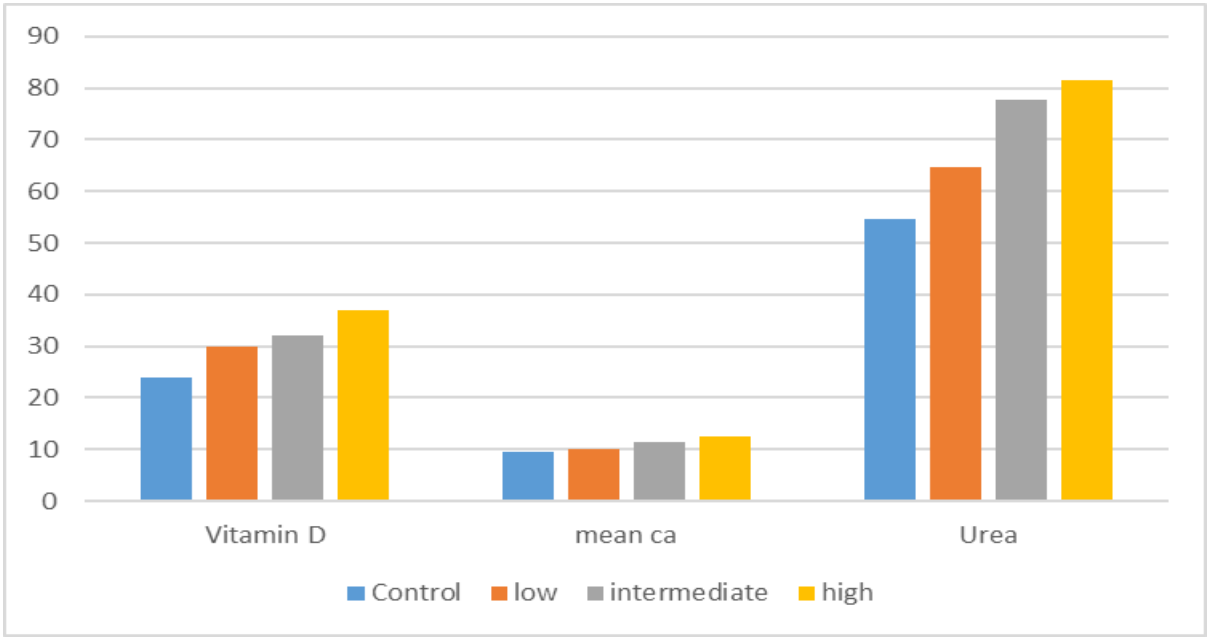

Figure (11). Measurements of calcium, blood nitrogen urea, and vitamin D levels in mice blood.

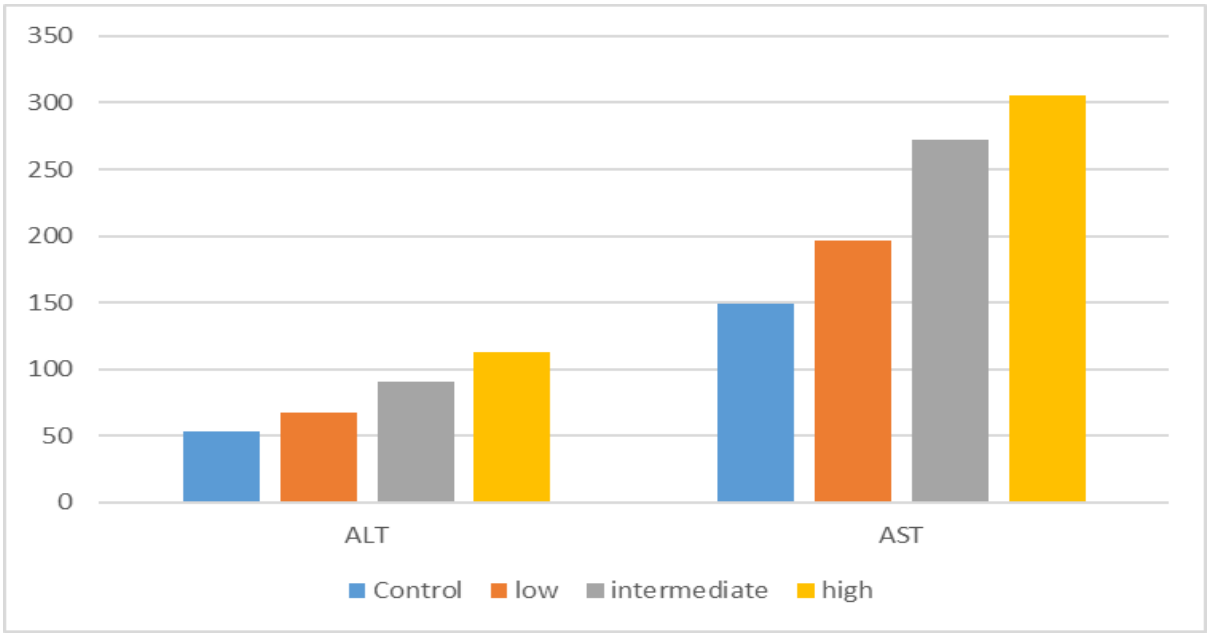

Figure (12). Measurements of liver ALT and AST enzymes.