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

Amr Abd El Nasser¹, Asmaa A. Abdelrahman², Bahaa E. Abd El-Fatah³, Rania M. Ewida¹, Ameer Elfarash²
¹Applied Biotechnology Dept., Molecular Biology Research & Studies Institute. Assiut University, Assiut, Egypt.
²Histology Dept., Faculty of Veterinary. Assiut University Assiut, Egypt.
³Genetics Dept., Faculty of Agric. Assiut University, Assiut, Egypt.
⁴Department of Food Hygiene, Faculty of Veterinary Medicine. New Valley University, New Valley, Egypt.
Corresponding author: Amr Abd El Nasser E: mail: Amr.Abd337@mbi.aun.edu.eg

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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.
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; Priel 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 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 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 1μm of vitamin D.
Group 3: intermediate dose group, every mouse was administrated with 1ml of corn oil which contains 2 μm of vitamin D.
Group 4: high dose group, every mouse was administrated with 1ml of corn oil containing 3 μm of vitamin D.

After 8 weeks, the mice were slaughtered 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 °C for long term storage or at -20 °C 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).

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 demonstared that Vitamin D3 supplementation...
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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 experiments.

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 fibroelastic 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 led 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, difficulty 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 severely haemorrhage in cells. This damage in liver cells led 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 led 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.

**REFERENCES**

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Tables: -

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5′→3′ sequence)</th>
<th>Reverse (5′→3′ sequence)</th>
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<tbody>
<tr>
<td>VDR</td>
<td>GAGGTGTCTGAAGCCTGGAG</td>
<td>ACCTGCTTTCTGGGTAGGT</td>
</tr>
<tr>
<td>CYP3A11</td>
<td>TCACACACACAGTTGAGGAGGA</td>
<td>GTCCATCCCTGCTTGTGTC</td>
</tr>
<tr>
<td>TRPV6</td>
<td>CTGCAAGGGTGGAGGAGGC</td>
<td>TACAGGCACCAGTCTAGGGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGACCTCAACTACATGGTCTACA</td>
<td>CTCCCATTCTCGGCCCTTG</td>
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</tbody>
</table>

Table 2: Means of heart weight in different groups

<table>
<thead>
<tr>
<th></th>
<th>Mean (mg)</th>
<th>LSD*</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>198.9</td>
<td>a</td>
</tr>
<tr>
<td>Low</td>
<td>210.0</td>
<td>b</td>
</tr>
<tr>
<td>Intermediate</td>
<td>217.4</td>
<td>b</td>
</tr>
<tr>
<td>High</td>
<td>231.8</td>
<td>c</td>
</tr>
</tbody>
</table>

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