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Dose-dependent effects of vitamin $1,25(OH)_2D_3$ on oxidative stress and apoptosis

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Abstract

Background: The purpose of this study is to examine the dose-dependent effects of vitamin $1,25(OH)_2D_3$ on apoptosis and oxidative stress.

Methods: In this study, 50 male Balb/c mice were used as control and experiment groups. The mice were divided into 5 groups each consisting of 10 mice. Calcitriol was intraperitoneally administered as low dose, medium dose, medium-high dose and high dose vitamin D groups (at 0.5, 1, 5 and 10 μg/kg, respectively), for three times a week during 14 days. At the end of the study, annexin V was measured by enzyme-linked immunosorbent assay method, and total antioxidant capacity and total oxidant status values were measured by colorimetric method in serum. Hematoxylin eosin staining was performed in liver tissues and periodic acid schiff staining was performed in kidney tissues.

Results: While comparing the results of medium-high dose (5 μg/kg) and high dose (10 μg/kg) vitamin D administration to that of the control group, it was observed that serum antioxidant status and annexin V levels decreased and glomerular mesenchial matrix ratio increased in kidney ($p < 0.05$). In addition to these findings, in the group receiving high dose vitamin D (10 μg/kg), it was observed that the damage to the liver increased together with the the oxidative stress index values ($p < 0.05$).

Conclusions: As a result, this study was the first in the literature to report that use of high-dose vitamin D (10 μg/kg) results in oxidant effect, rather than being an antioxidant, and causes severe histopathological toxicity in the liver and kidney.

Keywords: apoptosis; calcitriol; oxidative stress; total oxidant status; total antioxidant capacity.

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Introduction

Vitamin D is an important steroid hormone among fat-soluble vitamins [1, 2]. It plays a central role in the calcium and phosphorus hemostasis required for bone tissue calcification [3]. Vitamin D_2 is synthesized photochemically in plants, while vitamin D_3 is synthesized in dermis and epidermis by the effect of sunlight [4]. Vitamin D exists in the body in 25(OH)D and $1,25(OH)_2D$ forms.

$1,25(OH)_2D_3$ (calcitriol), which is the active form of vitamin D, binds to the vitamin D receptors (VDR) found in tissues and plays a role in the etiopathogenesis of diseases such as rickets and osteomalacia, which are related to the calcium metabolism, as well as autoimmune diseases, cardiovascular diseases, depression and cancer [5]. Besides, demonstration of VDR and 1-α hydroxylase (CYP27B1) enzyme in many tissues including pancreas, immunological system, macrophages, vascular endothelium, stomach, epidermis, colon, placenta, brain, and cancer cells implies that vitamin D might also have some unknown effects [6].

Apoptosis is an event of programmed cell death with different biochemical and genetic pathways that play a critical role in normal tissue development and homeostasis [7]. Calcitriol, which stimulates calcium entry, transfer, and calcium buffering in different cell types, plays a role in the regulation of the apoptosis process that determines the outcome of health and disease in the cell [8, 9]. Increasing intracellular calcium triggers apoptosis by triggering apoptosis signaling pathways [10]. Besides, calcitriol suppresses the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL and induces pro-apoptotic proteins Bax and Bak, likewise leading to cell apoptosis [11].

It is known that vitamin D affects oxidative stress. Vitamin D reduces the lipid peroxidation by stabilizing the plasma membrane. At the same time, it also exhibits antioxidant properties by enabling upregulation of antioxidant systems such as glutathione, glutathione peroxidase, and superoxide dismutase via nuclear receptors [12].

The deficiency of calcitriol, which has many functions, is widely observed. For this reason, the studies on vitamin D are mostly based on the effects of its deficiency. But in recent years, excessive calcitriol supplementation has resulted in vitamin D toxicity. However, the effects of high doses of vitamin D on apoptosis and oxidative stress are unknown [13, 14].

This study is intended to demonstrate the dose-dependent effects of calcitriol on apoptosis and oxidative stress.

Materials and methods

Animals

Some 50 healthy Balb/c type male mice aged 10 weeks were used. The experiment animals were taken to the laboratory 1 week before study to get accustomed to the environment. The mice were maintained in a 12-h light and 12-h dark environment and the ambient temperature was maintained at 22°C ± 1°C. Humidity of the medium was between 65% and 70%. There were no restrictions on nutrition and water (tap water) (ad-libitum). All respective procedures were approved by Istanbul Medipol University Ethics Committee of Experimental Animal Use and the Research Scientific Committee at the same institutions (IMU-HADYEK) (Protocol 38828770-604.01.01-E.12050).

Vitamin D administration

Each experimental group was divided into five groups of 10 mice each. Among the experiment groups, low dose vitamin D group was administered with 0.5 μg/kg, medium dose vitamin D group was administered with 1 μg/kg, medium-high dose vitamin D group was administered with 5 μg/kg, and high dose vitamin D group was administered with 10 μg/kg of vitamin D$_3$ Calcijex (Liscate, Italy) intraperitoneally, three times a week (Monday, Wednesday, and Friday) for 14 days. The control group was intraperitoneally administered with normal saline (Table 1) [15–17].

Three days after the administration of vitamin D was terminated, blood was drawn from the mice under anesthesia, taken into red-capped blood vessels by cardiac puncture, and sera were separated by centrifugation at 3500 rpm for 10 min [17]. Liver and kidney tissues were also taken for histopathological examination of mice.

Parameters and methods examined in blood samples

Total antioxidant capacity (TAC), total oxidant status (TOS), calcium and annexin V levels were measured in sera obtained from mice.

Table 1: Experiment groups and doses of calcitriol administered.

Group no.	Group name (n = 10)	Dose of calcitriol administered, μg/kg
1	Control group	Normal saline
2	Low dose vitamin D	0.5
3	Medium dose vitamin D	1
4	Medium-high dose vitamin D	5
5	High dose vitamin D	10

Calcium levels were measured using a Roche Cobas 6000 biochemical analyzer (Roche Diagnostic, USA). TAC, TOS, oxidative stress index (OSI) and annexin V enzyme-linked immunosorbent assay (ELISA) (Kit no: E0502Mo BT LABS, Shanghai, China) were conducted using a SpectraMax i3 Multimode Microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Annexin V

We worked on annexin V using Mouse annexin V ELISA kit (Kit no: E0502Mo), a product of Bioassay Technology Laboratory.

This kit measures mouse annexin V using a double antibody sandwich technique. In this method, annexin V antigen is added to the wells previously coated with annexin V monoclonal antibody, and then incubated. Then streptavidin-horseradish peroxidase is added and anti-annexin V labeled with biotin is added. Afterwards, standard ELISA procedure is followed. The intensity of the color, which is proportional to the concentration, is read.

Total antioxidant assay

The TAC value was measured colorimetrically in serum using the method developed by Erel [18]. ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma-Aldrich, Taufkirchen, Germany) reagent is radicalized by hydrogen peroxide (Sigma-Aldrich, Taufkirchen, Germany). When serum is added, antioxidants in the serum neutralize existing ABTS radicals. The absorbance is measured at 658 nm.

Total oxidant assay

The TOS value was measured colorimetrically in serum using the method developed by Erel [19]. Fe_2SO_4 dissolves in water, releasing Fe^{2+}. Oxidants found in serum enable Fe^{2+} to Fe^{3+} oxidation. The X-orange (Sigma-Aldrich, Taufkirchen, Germany) reagent used gives a colored complex with Fe^{3+}. The absorbance is measured at 658 nm. OSI is calculated by the formula (TOS/TAC) × 100.

Histopathological analyses

The kidneys and the livers removed from the mice were fixed in 10% neutral buffered formalin (Sigma-Aldrich, Taufkirchen, Germany). Then these were embedded in paraffin using routine light microscopy follow-up methods. Paraffin sections taken at 5 mm thickness

with the aid of a microtome (Thermo Microm HM 340E, Waltham, MA, USA) were placed in the slides to perform light microscopy. Periodic acid Schiff (PAS) and hematoxylin-eosin (HE) staining methods were applied to the tissue sections.

PAS staining: Changes in the structure of the mesenchial matrix were identified by PAS staining. In PAS-stained kidney tissue sections, the glomerular mesenchial matrix ratio was performed by double-blind study on 20 regular shaped glomeruli selected from random fields at the ×40 magnification using a Nikon Eclipse Ni research microscope (Nikon Instruments Inc., Melville, NY, USA). The rate of lesion in each glomerulus ranged from 0 to + 4. 0: mesenchial matrix ratio is normal; +1: 25% increase in mesenchial matrix; +2: 50% increase in mesenchial matrix; +3: 70% increase in mesenchial matrix; + 4: 100% increase in mesenchial matrix.

The values recorded according to this scoring were collected separately for each slide. For example: of the 20 glomeruli, 5 were +3, 10 were +2, and 5 were +1. These results were incorporated into the formula in the literature and the results were obtained for each slide. When incorporating the above values in the formula: $(3\times5/20)+(2\times10/20)+(1\times5/20)\times100=125$ the obtained number was recorded as mesenchial matrix damage score [20].

HE staining: Changes in the structure of liver tissue were shown by HE staining. Slides were examined under light microscope at ×50 and ×100 magnification and histopathological scoring was performed.

Grade 0: Minimal or no evidence of damage.

Grade 1: Cytoplasmic vacuolization characterized by mild damage, together with focal nuclear picnosis.

Grade 2: Moderate damage, mild mononuclear cell infiltration and cytoplasmic vacuolization, as well as hepatocyte swelling, no necrosis, sinusoidal dilatation and congestion, turbidity at the intercellular border.

Grade 3: Coagulation necrosis, severe mononuclear cell infiltration, cytoplasmic hypereosinophilia, extensive sinusoidal dilatation, and congestion in mild damage.

Grade 4: Severe coagulation necrosis in severe damage, disintegration and hemorrhage in hepatic cell cords, deterioration of tissue structure [21].

Statistical analysis

The Windows operating system IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA) program was used to evaluate the statistical analysis of the data obtained from 50 Balb/c mice. In this study, annexin V, TAC, OSI, calcium, body weight at days 0, 7 and 14, kidney glomerulus mesenchial matrix ratio and liver histopathological scoring values were compared among the experiment groups, to which 0.5, 1, 5, and 10 μg/kg calcitriol were administered, and correlation analysis was made. The normal distribution suitability of the parameters was investigated using the Shaphiro-Wilk test. Differences in groups suitable for normal distribution were assessed using the one-way ANOVA test, while unsuitable groups were assessed using the Kruskall-Wallis test. Statistical significance level was accepted as $p<0.05$. Spearman and Pearson correlation analysis was used to investigate the relationship between the parameters.

Results

Calcium levels and body weight

Calcium levels were measured at the end of vitamin D administration. The calcium level of the control group was 9.12 ± 0.36 mg/dL and the calcium levels of low, medium, medium-high, and high-dose groups were 9.29 ± 0.59, 9.20 ± 0.37, 9.06 ± 0.57; 9.19 ± 0.38 mg/dL, respectively. There was no statistically significant difference between the control group and the experimental groups in terms of calcium level ($p>0.05$) (Table 2). Between the control

Table 2: Comparison of laboratory findings of control and dose groups.

Parameters	Control group (1)	Low dose vitamin D group (2)	Medium dose vitamin D group (3)	Medium-high dose vitamin D group (4)	High dose vitamin D group (5)	p-Value	Intergroup[a] significance
Calcium, mg/dL	9.12 ± 0.36	9.29 ± 0.59	9.20 ± 0.37	9.06 ± 0.57	9.19 ± 0.38	> 0.05	–
TAC (mmol Trolox eq.)	2.23 ± 0.41	2.14 ± 0.43	1.89 ± 0.40	1.51 ± 0.24	1.56 ± 0.37	< 0.05	4.1 5.1 2.4 2.5
TOS (μmol H_2O_2)	28.36 ± 7.26	25.66 ± 4.92	24.29 ± 5.65	22.53 ± 3.10	28.01 ± 7.37	> 0.05	–
OSI	12.80 ± 2.38	12.11 ± 1.52	13.05 ± 2.45	15.11 ± 2.05	18.38 ± 5.16	< 0.05	5.1 5.3 5.4
Annexin V, ng/mL	13.44 ± 1.02	11.81 ± 1.26	11.26 ± 1.32	10.56 ± 2.23	11.06 ± 1.84	< 0.05	1.3 1.4 1.5

$p<0.05$ was considered statistically significant. [a]Groups (Groups 1–5) of statistical difference are stated. TAC, total antioxidant capacity; TOS, total oxidant status; OSI, oxidative stress index.

group and the experimental group, there was no statistically significant difference in the body weights of Balb/c mice at 0, 7, and 14 days ($p > 0.05$).

Annexin V

When compared with the control group (13.44 ± 1.02 ng/mL), the annexin V values (11.26 ± 1.32, 10.56 ± 2.23, 11.06 ± 1.84 ng/mL, respectively) in the medium dose, medium-high dose, and high dose groups were determined to be low at a statistically significant level ($p < 0.05$) (Table 2). In addition, there was a positive correlation between annexin V and TAC and TOS ($p < 0.01$).

TAC, TOS, and OSI examination

When the TAC values were examined, the values of the medium-high dose and high-dose vitamin D groups (1.51 ± 0.24, 1.56 ± 0.37 mmol, respectively) were found to be statistically lower compared to the control group (2.23 ± 0.41 mmol) (Table 2).

Also, the TAC values of the medium-high dose and high-dose vitamin D groups were statistically lower than the TAC values of the group receiving low-dose vitamin D (2.14 ± 0.43 mmol) ($p < 0.05$) (Table 2). There was a positive correlation between annexin V and TAC and TOS ($p < 0.01$).

When the TOS values of the control group and the experimental group were compared, it was found that there was no statistically significant difference ($p > 0.05$) (Table 2).

Although OSI values exhibited a progressively increasing numeral trend in all groups according to the control group (12.80 ± 2.38), only the high-dose (18.38 ± 5.16) vitamin D group was found to be higher at a statistically significant level as compared to the control group (12.80 ± 2.38), medium dose (13.05 ± 2.45), and medium-high dose (15.11 ± 2.05) vitamin D groups ($p < 0.05$) (Table 2) (Figures 1–3). There was also a significant negative correlation between OSI and TAC ($p < 0.01$). There was a significant positive correlation between OSI and TOS ($p < 0.01$).

PAS staining in kidney

In the PAS staining, the kidney glomerulus mesenchial matrix ratio was found to be higher at a statistically significant level in the high dose (159.2 ± 51.7) and medium-high dose (159.2 ± 51.7) calcitriol experiment groups as

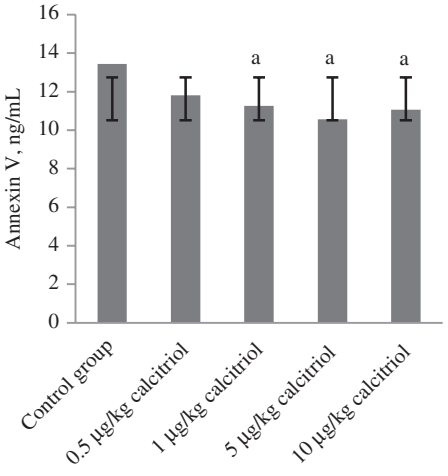

Figure 1: The effect of calcitriol on annexin V levels at various doses.
Values are expressed as mean ± SD for the involved 10 mice in each group. [a]Significantly different when compared with the control group at $p < 0.05$.

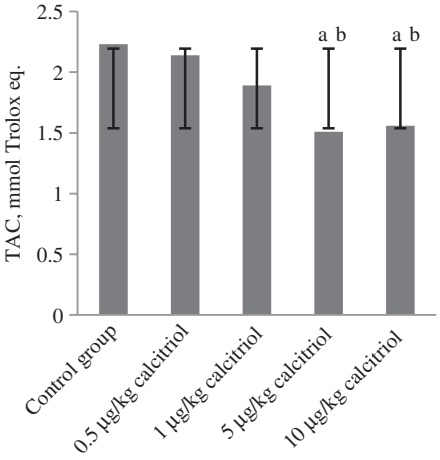

Figure 2: The effect of calcitriol on TAC levels at various doses.
Values are expressed as mean ± SD for the involved 10 mice in each group. [a]Significantly different when compared with the control group at $p < 0.05$. [b]Significantly different when compared with the low dose vitamin D group at $p < 0.05$.

compared to the control (7.80 ± 7.50), low dose (14.5 ± 4.97), and medium dose (14.50 ± 4.97) groups ($p < 0.05$) (Table 3).

Mesenchial matrix ratio, parathyroid leaf cells, and bowman capsule of the mice in the control group, low-dose vitamin D group, and medium-dose vitamin D group were observed to be in normal structure (Figure 4C). In the medium-high dose and high-dose vitamin D groups, increase in mesenchial matrix ratio, hypertrophy and hyperplasia in parietal leaf cells and bowman capsule interval was observed (Figure 4D, E).

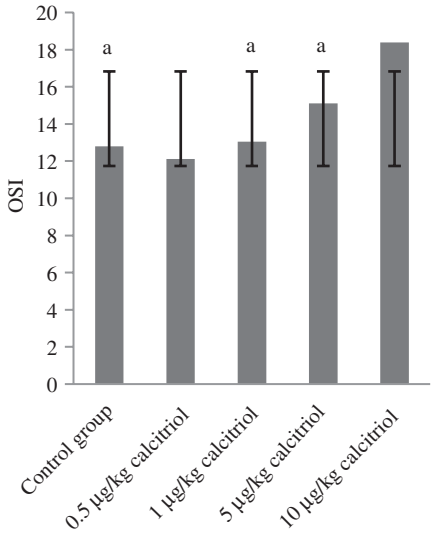

Figure 3: The effect of calcitriol on OSI levels at various doses. Values are expressed as mean ± SD for the involved 10 mice in each group. [a]Significantly different when compared with the high dose vitamin D group at $p < 0.05$.

In kidneys, a positive correlation was observed between glomerulus mesenchial matrix ratio and OSI ($p < 0.05$).

HE staining in liver

HE staining and histopathological scoring on the liver revealed increasing numerical values towards high doses. However, only high-dose (1.1 ± 0.57) vitamin D group had a statistically significant higher value than the control group (0.3 ± 0.48) ($p < 0.05$) (Table 3).

For the mice in the control group, low-dose and medium-dose vitamin D groups, it was observed that the structures of the central venous structure, sinusoidal spaces, and radial hepatocyte cords in the liver tissue of the mice were normal (Figure 5A–C). In the medium-dose D vitamin group, no abnormal expansions were observed in the central vein (Figure 5D). In the high-dose D vitamin group, it was observed that the central vein lost its inherent structure, the clearances of sinusoidal cavities increased, and the radial hepatocyte cords became irregular (Figure 5E).

A negative correlation was observed between liver histopathological scoring and TAC ($p < 0.01$) and annexin ($p < 0.05$). A positive correlation was observed between liver histopathological scoring and OSI ($p < 0.01$).

Discussion

This study aims to demonstrate the dose-dependent effects of calcitriol on apoptosis and oxidative stress.

In addition to the calcium-dependent effects of vitamin D, there are also calcium-independent effects on carcinogenesis, immunological function, autoimmune diseases, and cardiovascular diseases. Furthermore, vitamin D compounds are known to have effects on proliferation, differentiation, apoptosis, and cell cycle [22, 23]. In the kidney and liver, vitamin D is believed to play anti-inflammatory and anti-fibrotic roles by down-regulating production of tumor necrosis factor α and transforming growth factor β by binding to regulators of target genes [24].

Cell culture studies on the relation between calcitriol and apoptosis reveals apoptotic or anti-apoptotic effects of vitamin D. Annexin V, used to measure apoptosis, is a protein in the cell proliferation process with anti-inflammatory and anticoagulant properties found in lung,

Table 3: Comparison of histopathological findings of control and experimental group.

Parameters	Control group (1)	Low dose vitamin D group (2)	Medium dose vitamin D group (3)	Medium-high dose vitamin D group (4)	High dose vitamin D group (5)	p-Value	Intergroup[a] significance
Mesenchial matrix ratio in kidney (%)	7.80 ± 7.50	14.5 ± 4.97	14.50 ± 4.97	129.5 ± 33.3	159.2 ± 51.7	<0.05	5.1 5.2 5.3 4.1 4.2 4.3
Histopathological scoring in liver (%)	0.3 ± 0.48	0.7 ± 0.48	0.7 ± 0.48	0.8 ± 0.42	1.1 ± 0.57	<0.05	5.1

$p < 0.05$ was considered statistically significant. [a]Groups (Groups 1–5) of statistical difference are stated.

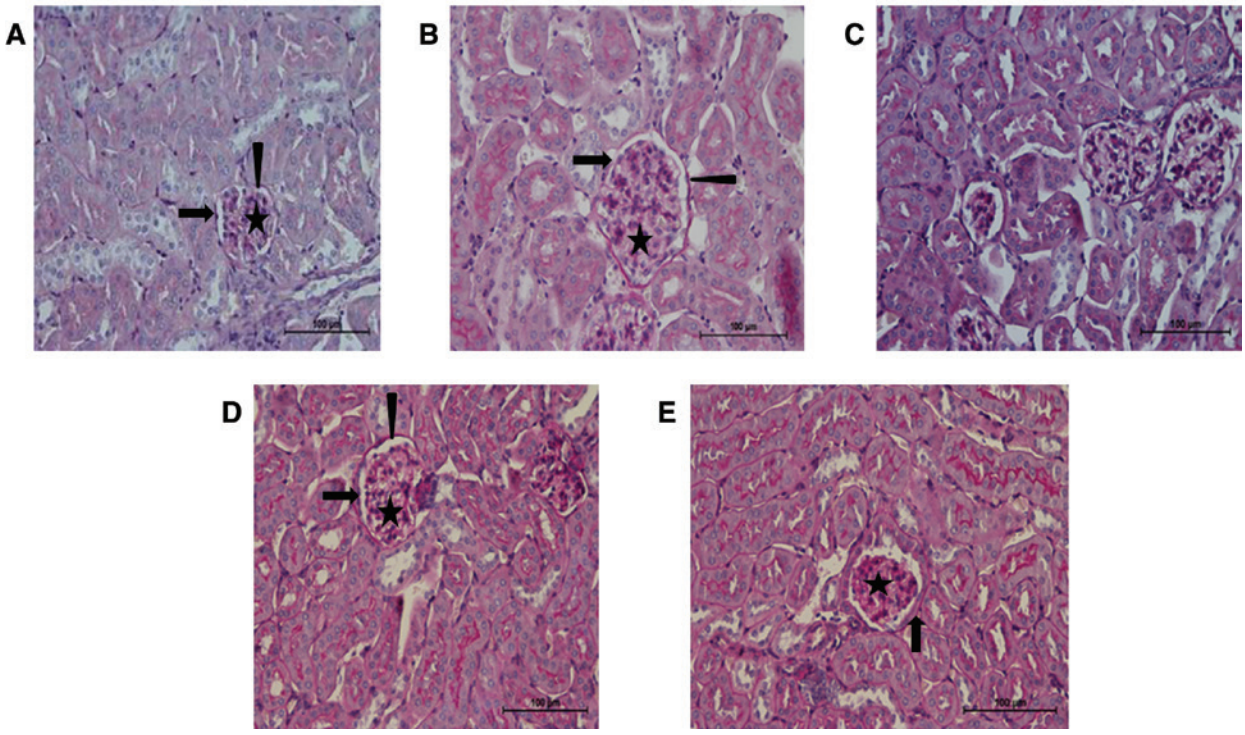

Figure 4: PAS staining in kidney tissue.
Parietal leaf cells are shown with arrow, bowman capsula interval with star, and mesenchial matrix with triangle symbols. (A) Control group; (B) low dose vitamin D group; (C) medium dose vitamin D group; (D) medium-high dose vitamin D group; and (E) high dose vitamin D group.

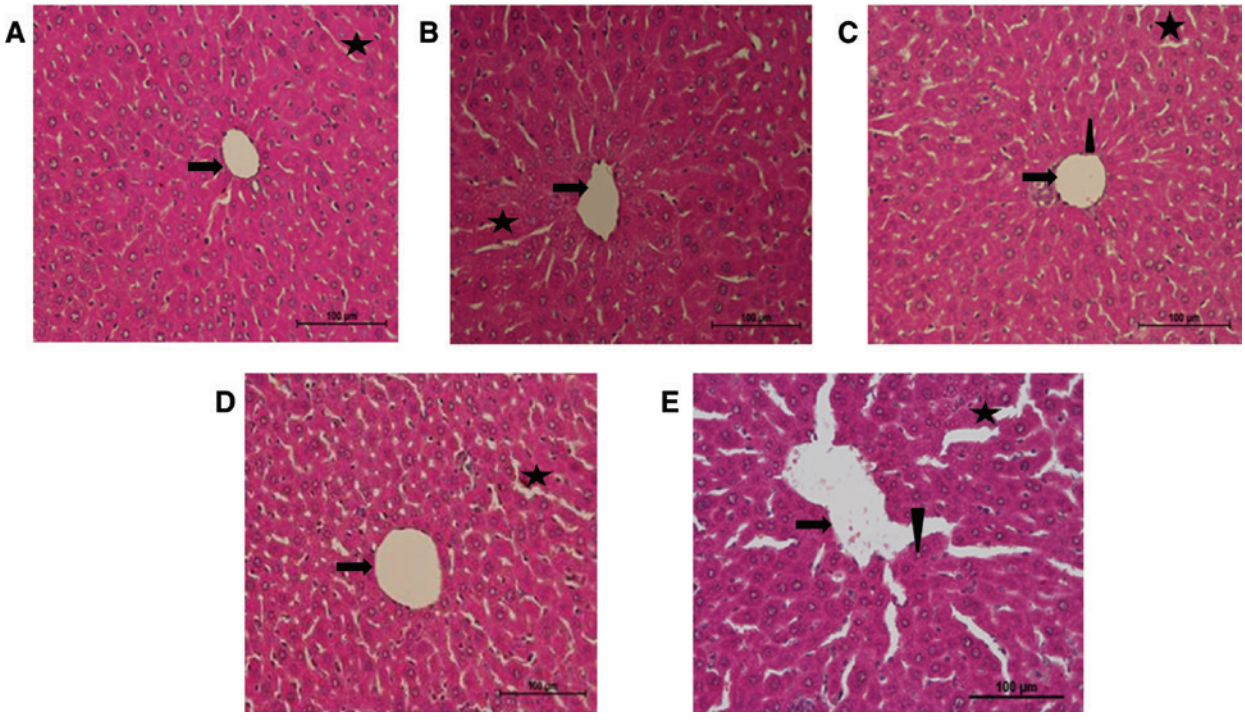

Figure 5: Immunohistochemical staining with HE in the liver.
Central vein is shown with arrow, sinusoidal spaces with star, and radial hepatocyte cords with triangle symbols. (A) Control group; (B) low dose vitamin D group; (C) medium dose vitamin D group; (D) medium-high dose vitamin D group; and (E) high dose vitamin D group.

kidney, liver, and skeletal muscles [25]. Some studies examining the association between apoptosis and vitamin D revealed that calcitriol induces apoptosis [26–30]; the Morales et al. study has shown that vitamin D suppresses apoptosis [31]. It is assumed that the level of annexin V in the serum theoretically tends to be low in the beginning of apoptosis, and increases with increasing apoptosis. The low level of annexin V in this study can be explained by the fact that apoptosis is in the initial phase. In this study, a strong positive correlation was found between annexin V and TAC and TOS values.

Oxidative stress is known to cause more than 100 diseases, and the antioxidant mechanism plays an important role in preventing them. Vitamin D isoforms 7-dehydrocycalciferol, cholecalciferol, vitamin D_2, and calcitriol may exhibit antioxidant properties by reducing lipid peroxidation [32]. In addition, there are studies which show that vitamin D reduces oxidative stress by increasing antioxidants [33–35]. Some studies suggest that vitamin D has no effect on oxidative stress [36].

When the OSI values that measure oxidative stress better than TAC and TOS were examined in this study, a progressively increasing numeral trend was observed between the control and high dose groups. However, statistically, only the OSI values of the group receiving high-dose vitamin D were found to be higher than that of the control, medium, and medium-high vitamin D groups ($p < 0.05$).

In addition, there was a positive correlation between OSI and TOS values and a negative correlation between OSI and TAC values, as expected in the study.

Calcitriol is responsible for regulating the calcium level in the body [37]. However, high doses of vitamin D supplementation are toxic and cause hypercalcemia. Hypercalcemia is defined as a common and potentially life-threatening condition [38]. Studies on the dose-response relation of vitamin D have shown that administration of calcitriol at certain doses leads to hypercalcemia and weight loss [39, 40]. In this study, it was observed that the calcitriol administered to Balb/c mice does not alter calcium levels. This can be attributed to the short duration of vitamin D administration. No weight loss was observed in this study, which is expected as a result of hypercalcemic effect. Calcium homeostasis may be achieved due to blood withdrawal from the mice 3 days after terminating the administration of vitamin D. Similar to the study by Ajibade et al. on C57BL/6 mice, it was shown that calcitriol does not lead to hypercalcemia and weight loss [41]. When the glomerular basal membrane and mesenchial matrix ratios were examined histopathologically with PAS staining, no abnormal findings were found in the kidney sections of control, low-dose, and medium-dose vitamin D groups.

Mesenchial matrix increase was observed in the kidney sections of medium-high dose and high-dose vitamin D treated groups. Also, hypertrophy and hyperplasia were observed in the bowman capsula parietal leaf cells and hypertrophy was observed in the glomeruli. This can be interpreted as high doses of vitamin D causing toxic effects in kidneys.

A statistically significant increase was observed in the histopathological score of HE-stained liver in the group receiving high dose (1.1 ± 0.57) vitamin D, compared to the control group (0.3 ± 0.48).

There were no abnormal findings in the liver sections of the control, low dose, and medium dose vitamin D groups. A slight degeneration of the liver parenchyma was observed in hepatic sections of medium-high dose and high dose vitamin D groups. In addition, it was observed that the sinusoids were dilated and the natural structure of central vein was lost. As a result, it can be said that medium-high and high doses of vitamin D damaged the histology of the liver.

In our study, we examined the effects of different vitamin D doses on apoptosis and oxidative stress and observed a statistically significant decrease in the serum TAC levels, decrease in the annexin V value, and increase in the glomerular mesenchial matrix ratio in the kidney after administration of medium-high dose vitamin D. In addition to these findings, it was seen that OSI value increased statistically significantly in the administration of high dose vitamin D, and histopathological damage in the liver occurred.

As a result, this study shows that the use of high dose vitamin D is that of an oxidant rather than an antioxidant, leading to severe histopathological toxicity in the liver and kidney.

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