

## Original Research Article

# Vitamin D: An effective therapy against methotrexate-induced cardiotoxicity

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### Abstract

**Purpose:** To determine the potential cardioprotective effect of vitamin D (VD) against methotrexate (MTX) induced cardiotoxicity.

**Methods:** A total of thirty-five (35) rats were randomly assigned to five equal groups. Control group received no treatment; MTX group received intraperitoneal (IP) injection of MTX in a single dose of 20 mg/kg on day 8; VD group received 200 IU/kg VD daily dissolved in sunflower oil orally; sunflower oil (SO) group received 1 mL/kg/day SO orally; MTX + VD group received a single dose of MTX (20 mg/kg, IP) on day 8 and VD (200 IU/kg, orally) for 21 days. Myocardial tissue samples were harvested and used for clinical chemistry, histopathological, and ultrastructural evaluation.

**Results:** Histopathological damage in MTX group was more severe than in control group under both light and electron microscopy. Expression of transient receptor potential melastatin 2 (TRPM2) and caspase-3 markers was significantly higher in MTX group ( $p < 0.05$ ). Glutathione peroxidase (GSH-Px) enzyme activity in cardiac tissue was lower in MTX group, whereas malondialdehyde (MDA) levels increased significantly ( $p < 0.05$ ). In MTX + VD group, VD treatment alleviated histopathological damage and significantly lowered TRPM2 and caspase-3 expressions ( $p < 0.05$ ). Vitamin D also reduced tissue MDA levels, and increased GSH-Px activity albeit non-significantly ( $p > 0.05$ ).

**Conclusion:** These findings suggest that VD exerts an ameliorative effect on MTX-induced cardiotoxicity in rats. Therefore, TRPM2 channel affords a novel therapeutic approach for treatment of diseases related to chemotherapy-induced oxidative stress.

**Keywords:** Cardiotoxicity, Caspase-3, Methotrexate, TRPM2, Vitamin D

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## INTRODUCTION

Methotrexate (MTX) has cytotoxic activity and folate antagonistic effect and is prescribed for treating cancers and autoimmune diseases. The cytotoxic effect of MTX is not limited to tumor cells, it also impacts essential organs including the heart [1]. Methotrexate inhibits DNA

synthesis and cell proliferation by inhibiting folic acid reduction. On the other hand, clinical research has shown that the drug also has antiproliferative properties and causes direct cellular damage [2].

Transient receptor potential (TRP) channels act like cation channels. They not only depolarize the

membrane potential but also regulate intracellular cation concentrations such as calcium ions ( $\text{Ca}^{2+}$ ). They also serve as “biosensors”, sensing changes in the environment and cellular circumstances. Transient receptor potential melastatin 2 (TRPM2), a member of the TRP superfamily, is a widely distributed non-selective  $\text{Ca}^{2+}$  permeable cation channel. Reactive oxygen species (ROS) activates TRPM2 channel, causing it to increase intracellular  $\text{Ca}^{2+}$  concentrations, and  $\text{Ca}^{2+}$  entry into the cell has been identified as the beginning of pathophysiological processes leading to cell death [3,4]. Vitamin D (VD) has generated a large body of literature in the recent decade, not only for its well-known activity as a steroid hormone on skeletal tissue but also for its possible beneficial effect on the cardiovascular system. Cardiomyocytes, as well as fibroblasts, vascular endothelial, and smooth muscle cells, express VD receptors (VDR) and hydroxylases required for the active form of VD. Vitamin D prevents cellular hypertrophy and regulates the renin-angiotensin-aldosterone system by pleiotropic effects [5]. Nonetheless, VD has antioxidant, anti-inflammatory, anti-fibrotic, and anti-atherosclerotic properties in cardiac metabolism [6]. Previous research revealed that it is a protective factor against cardiovascular disease by reducing oxidative stress, apoptotic and inflammatory pathways, and restoring cardiac integrity [7].

This study investigated the tendencies of VD to protect against myocardial toxicity caused by MTX in rats by analyzing histopathological, immunohistochemical, and biochemical parameters as well as electron microscopy.

## EXPERIMENTAL

### Animals

Throughout the experiment, researchers followed the rules established by the Principles for Laboratory Animal Care [13]. Ethical approval for this study was obtained from the Animal Research Ethics Committee of Kahramanmaraş Sutcu Imam University, (approval no. 2022/04-02). The effect of VD on cardiotoxicity due to MTX was studied in thirty-five adult male Wistar rats (200 – 300 g). The experiment was initiated after 7 days of acclimatization in ambient settings, with a 12 h light and 12 h dark cycle at constant temperature ( $22 \pm 3 \text{ }^\circ\text{C}$ ).

### Study design

Five groups of seven rats each were randomly selected from among the animals. The control

group received no treatment; MTX group received an intraperitoneal (IP) injection of MTX (Methotrexate®, Kocak Farma, Turkey) at a single dose of 20 mg/kg on day 8 [8]; VD group daily administration of 200 IU/kg VD (Devit-3® Deva, Turkey), dissolved in sunflower oil orally for 21 days [9]; sunflower oil (SO) group received daily administration of 1 mL/kg SO orally for 21 days; MTX + VD group received a single dose of MTX (20 mg/kg, IP) on day 8 and VD (200 IU/kg, orally) for 21 days.

Rats were sacrificed after 21 days and myocardial tissues analyzed for histopathology, transmission electron microscopy (TEM), immunohistochemistry, and biochemical parameters were evaluated.

### Histopathological studies

Following the fixation with 10 % formalin in phosphate buffer, ventricular myocardial tissues were dehydrated in increasing ethanol series and clarified in xylene before being embedded in paraffin. A rotary microtome (Leica, 2125RT) was used to cut 5- $\mu\text{m}$  thick slices from the paraffin blocks. Afterward, they were stained by Hematoxylin and Eosin (H & E) and evaluated using a Carl Zeiss Axio Imager A2 light microscope. Histopathological scoring of cardiac tissue was performed [8].

### Transmission electron microscopy examination (TEM)

The ventricular tissue samples were placed in freshly prepared 2.5 % glutaraldehyde for fixation and postfixed with 1 % osmium tetroxide. Following dehydration in ascending grades of ethanol, samples were cleared with propylene oxide and embedded in an open. The ultra-thin sections were obtained using an ultramicrotome (UCT-125; Leica Microsystems, GmbH, Vienna, Austria) and stained with uranyl acetate and lead citrate. Finally, stained sections were examined with a TEM (JEM-1011; JEOL Ltd, Tokyo, Japan).

### Immunohistochemical staining of TRMP2 and caspase-3

Sections were deparaffinized, rehydrated, and then boiled in a microwave in citrate buffer solution (pH: 6.0). The cooling period was followed by washing the sections with phosphate-buffered saline (PBS), incubating them with 3 % hydrogen peroxide solution and then treating with Ultra V Block (TA-125-UB; Thermo Fisher Scientific). Afterward, the sections were incubated with anti-TRPM2 (1:200, bs-

2888R, Bioss) and anti-caspase-3 (1:200, ab184787, Abcam) overnight at 4 °C. Following this, the sections were incubated with biotinylated goat anti-polyvalent (TP-125-BN, Thermo Fisher Scientific) for 10 min at room temperature. The slides were washed with PBS and incubated with streptavidin peroxidase (TS-125-HR, Thermo Fisher Scientific). Staining was completed with chromogen and after washing with PBS, the slides were counterstained with Mayer's hematoxylin for 2 min. The prepared sections were examined with a Leica DM500 light microscope and then photographed (Leica DFC295). The histoscore, which represents the prevalence of TRMP2 and caspase-3 immunoreactivity in myocardial tissue, was determined using the following scoring: 0.1: < 25 %, 0.4: 26 – 50 %, 0.6: 51 – 75 %; 0.9: 76 – 100 %, and intensity of immunoreactivity as 0: unstained, +0.5: very low, +1: low, +2: moderate, +3: severe. The prevalence multiplied by the staining intensity was used to calculate the score.

### Biochemical analysis

#### Malondialdehyde (MDA) level in heart tissue

Total MDA, a measure of lipid peroxidation, was analyzed according to the method of Ohkawa *et al* [10]. Results from MDA were presented as nanomoles per gram of wet tissue (nmol/g wet tissue).

#### Glutathione peroxidase (GSH-Px) in heart tissue

The activity of GSH-Px was measured using Beutler method [11]. The function of GSH-Px is to catalyze the oxidation of reduced glutathione (GSH) into oxidized glutathione (GSSG) using  $H_2O_2$ . When t-butyl hydroperoxide and  $H_2O_2$  are both present, the GSSG formed by GSH-Px is reduced to GSH with the assistance of glutathione reductase and NADPH. GSH-Px activity was measured using the spectrophotometric change in absorbance at 340 nm as the oxidation of NADPH to NADP occurs.

### Statistical analysis

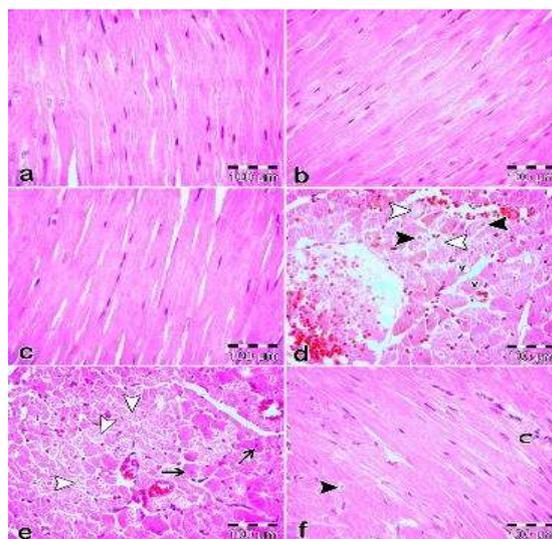
SPSS version 25.0 software package (IBM Inc, Chicago, IL) was used for data analysis. The Shapiro–Wilk test was used to determine whether the data was distributed normally. For comparing groups, one-way ANOVA or the Kruskal Wallis H tests were applied as required. Following these analyses, the significant differences were investigated using Tukey's test or the Mann-Whitney U test. Bonferroni

correction was used after non-parametric testing ( $P_{\text{adjust}} = \alpha/5C2 = 0.05/10 = 0.005$ ). The data are reported as mean  $\pm$  SD and median (minimum-maximum). Mean differences were considered significant at  $p < 0.05$ .

## RESULTS

### Histopathological features

The histoarchitecture of myocardial tissues in the control (Figure 1 A), SO (Figure 1 B), and VD (Figure 1 C) groups was normal, with central oval nuclei and acidophilic sarcoplasm. MTX group showed degenerative changes such as myofibril disruption, myocardial vessel congestion, focal hemorrhage, perinuclear vacuolization, degenerated myocytes with homogenized and intensely eosinophilic sarcoplasm and cellular vacuolization compared to the control group (Figure 1 D and 1 E). In MTX + VD group, features include cellular infiltrates, patches of disorganized myocardial fibers, less perinuclear vacuolization, and restoration of normal cardiac histoarchitecture in comparison to MTX group (Figure 1 F). The MTX group demonstrated considerably higher histopathological scoring than control and treatment groups, as shown in Table 1 ( $p < 0.001$ ). Moreover, MTX + VD group experienced significantly less myocardial damage than MTX group ( $p < 0.001$ ).



**Figure 1:** Photomicrographs of myocardial tissue sections by H&E (400 $\times$ ). (a) Control, (b) SO, and (c) VD groups. (d, e) MTX group; disruption of myofibrils (white arrowheads), perinuclear vacuolization (black arrowheads), degenerated myocytes with homogenized and intensely eosinophilic sarcoplasm (black arrows), and vacuolation (v) in the sarcoplasm. (f) MTX+VD group; perinuclear vacuolization (black arrowhead) and some cellular infiltrations (curved arrow)

**Table 1:** Histopathologic score of myocardial damage

Group	Median (min-max)
Control	0 <sup>a</sup> (0-1)
SO	0 <sup>a</sup> (0-1)
VD	0 <sup>a</sup> (0-1)
MTX	3 <sup>b,c</sup> (2-4)
MTX+VD	2 <sup>b</sup> (0-3)
<i>P</i> <sup>⊖</sup> values	< 0.001

<sup>⊖</sup>: Kruskal Wallis H test was used. Mann Whitney U Test was used for pairwise comparisons. <sup>a</sup>: No significant difference between control, SO, and VD groups ( $p > 0.05$ ). <sup>b</sup>: Significant difference between control, SO, VD, MTX, and MTX+VD groups ( $p < 0.001$ ). <sup>c</sup>: Significant difference between MTX and MTX+VD groups ( $p < 0.001$ )

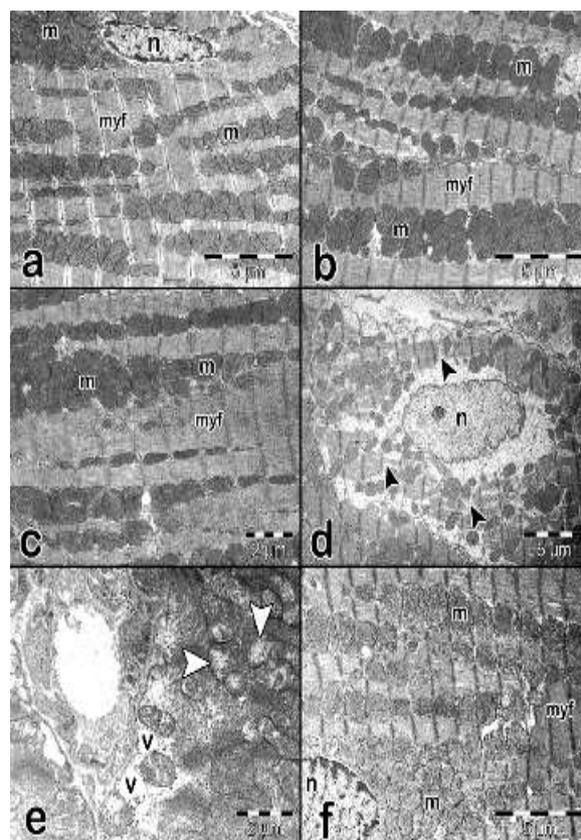
### Electron microscopic evaluation

Electron microscopy of ventricular myocardial cells from the control (Figure 2 A), SO (Figure 2 B), and VD (Figure 2 C) groups revealed typical morphological characteristics such as cardiomyocytes with a euchromatic oval nucleus, bundles of myofibrils arranged in light and dark bands, and numerous mitochondria around the nucleus and in regular rows between myofibrils. Cardiomyocytes with indented nuclei, areas of myofibrillar loss, ruptured mitochondrial cristae, and vacuoles were observed in MTX-treated group compared to the control group (Figures 2 D and 2 E). Unlike MTX group, MTX + VD group had typical cardiomyocyte ultrastructure, including a euchromatic nucleus, numerous mitochondria in the perinuclear area, and a nearly normal myofibril arrangement (Figure 2 F).

### TRPM2 and caspase-3 expressions

The effects of MTX and VD on TRPM2 and caspase-3 expression are shown in Table 2. TRPM2 and caspase-3 expression were identical in the control, SO, and VD groups ( $p > 0.05$ ). Furthermore, TRPM2 (Figure 3 D) and caspase-3 (Figure 4 D) immunoreactivity increased in MTX group when compared to control group ( $p < 0.05$ ) but lower in MTX+VD group, which was statistically significant, compared to MTX group ( $p < 0.05$ ).

The control, SO, and VD groups' sections stained with TRPM2 and caspase-3 antibodies revealed a few positive cells (Figure 3 A, 3B, 3C; Figure 4A, 4B, 4C respectively). The intensity of TRPM2 (Figure 3 D) and caspase-3 (Figure 4 D) immunoreactivity was considerably higher in MTX group than control group ( $p < 0.05$ ). Intensity of caspase-3 immunoreactivity was reduced after MTX+VD administration ( $p < 0.05$ ; Table 2).



**Figure 2:** Transmission electron micrograph of the ventricular myocardial tissue. (a) Control group (7,500x); (b) SO group (7,500x); and (c) VD group (7,500x). Normal cardiomyocyte structure with an euchromatic oval nucleus (n), mitochondrion (m), and myofibrils (myf). (d) (5,000x); (e) MTX-treated group (12,000x); a cardiomyocyte with an indented nucleus (n), degeneration in myofibrils (black arrowheads), vacuoles (v), and mitochondrial degeneration (white arrowheads). (f) MTX+VD group

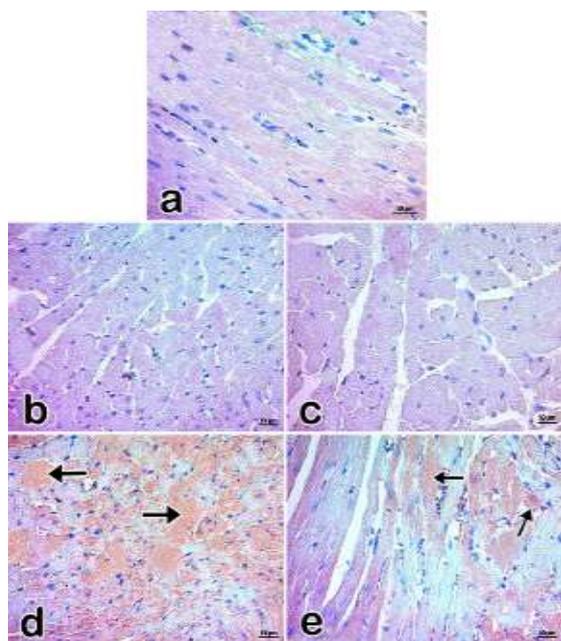
**Table 2:** Immunohistochemical analyses of all groups for TRPM2 and caspase-3 expressions

Group	TRPM2	Caspase-3
Control	0.60 <sup>c</sup> ±0.09	0.84 <sup>c</sup> ±0.09
SO	0.69 <sup>c</sup> ±0.09	0.69 <sup>c</sup> ±0.09
VD	0.65 <sup>c</sup> ±0.08	0.67 <sup>c</sup> ±0.08
MTX	2.04 <sup>a</sup> ±0.19	2.11 <sup>a</sup> ±0.19
MTX+VD	1.16 <sup>b</sup> ±0.10	1.35 <sup>b</sup> ±0.10
<i>P</i> * values	< 0.001	< 0.001

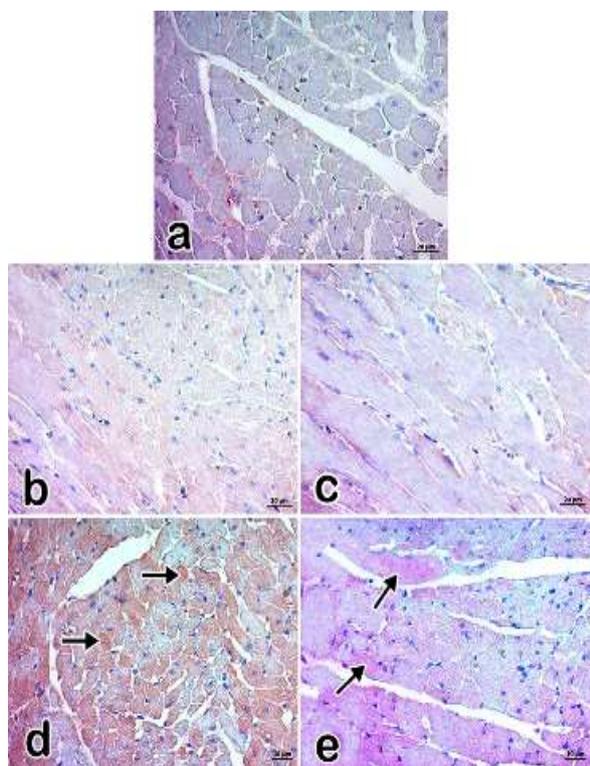
Data are expressed as mean ± SD. <sup>a, b, c</sup>. Means in same row with different superscripts differ significantly ( $p < 0.05$ , Tukey's test) \*One way ANOVA

### Effects of MTX and VD on MDA level

MTX group had higher cardiac MDA levels than the control group ( $p < 0.05$ ). Comparing MTX+VD group to MTX group revealed that although VD treatment decreased MDA levels, this difference was non-significant ( $p > 0.05$ ) (Table 3).



**Figure 3:** TRPM2 immunoreactivity image under a light microscope in rat ventricular myocytes (200 $\times$ ). (a) Control group, (b) SO group, and (c) VD group sections show few positive cells. (d) MTX group shows intense TRPM2 expression (arrows). (e) MTX+VD group shows mild TRPM2 expression (arrows)



**Figure 4:** Caspase-3 immunoreactivity image under light microscope in rat ventricular myocytes (200 $\times$ ). (a) Control group, (b) SO group, and (c) VD group sections show few positive cells. (d) MTX group shows intense caspase-3 expression (arrows). (e) MTX+VD group shows mild caspase-3 expression (arrows).

### Effects of MTX and VD on GSH-Px activity

The GSH-Px activity in MTX group was significantly lower in control group ( $p < 0.05$ ). Furthermore, in MTX+VD group, VD application increased GSH-Px activity, but it was non-significant compared to MTX group ( $p > 0.05$ ) (Table 3).

**Table 3:** Effects of MTX and VD on MDA level and GSH-Px activity

Group	MDA (nmol/g)	GSH-Px (nmol/g)
Control	0.78 <sup>b</sup> ±0.24	0.576 <sup>a</sup> ±0.01
SO	1.02 <sup>ab</sup> ±0.40	0.528 <sup>ab</sup> ±0.03
VD	0.91 <sup>ab</sup> ±0.23	0.526 <sup>ab</sup> ±0.02
MTX	1.42 <sup>a</sup> ±0.41	0.512 <sup>b</sup> ±0.03
MTX+VD	0.94 <sup>ab</sup> ±0.38	0.533 <sup>ab</sup> ±0.05
P* values	0.034	0.025

Data are expressed mean  $\pm$  SD. <sup>ab</sup>Means in same row with different superscripts differ significantly ( $p < 0.05$ , Tukey's test). \*One-way ANOVA

### DISCUSSION

MTX is a commonly employed chemotherapeutic agent. Inflammation and oxidative stress have been linked to the onset of MTX toxicity [1]. Because MTX inhibits the enzyme dihydrofolate reductase, it causes changes in cell metabolism. This inhibits DNA synthesis, which reduces the amount of protein in the cells and consequently, activity of cells [2], leading to cytotoxicity [1]. In the present study, 20 mg/kg MTX caused significant histopathological damage to cardiac structure in MTX group, which corroborates earlier research results in rodents [1,8]. The electron microscopic findings of the current study including vacuoles, myofibrillar and mitochondrial damage in cardiomyocytes, supported cardiotoxicity on an ultrastructural level, which was similar to earlier research [12,13]. The MTX causes generation of ROS and this leads to mitochondrial injury, resulting in lower mitochondrial adenosine triphosphate, mitochondrial matrix swelling, and cytochrome c release [14].

As oxidative stress is associated with several diseases, the development of effective therapeutic alternatives such as antioxidants, is highly important for preventing cardiovascular diseases [15]. Several *in vivo* and *in vitro* studies showed that Vitamin D (VD) has significant cardioprotective actions against acute myocardial infarction [16] and ischemia-reperfusion-induced cardiac injury [17]. The results revealed that VD treatment improved the histomorphology of cardiac tissue in both light and TEM examinations by mitigating mitochondrial degeneration and infiltration of inflammatory cells

consistent with earlier findings [16,17]. This regenerative ability could be attributed to anti-oxidative potential of VD, which is achieved by attenuating oxidative stress [7].

It is well-established that, there is a potential relationship between anticancer-induced apoptosis and oxidative stress in the heart [18]. In the current study, MTX-treated rats showed substantially increased expression of caspase-3 in cardiac tissue. These results were supported by several studies where MTX injection was linked to increased apoptosis [1,13,14]. The most likely trigger of apoptosis is excessive ROS generation after MTX exposure that results in mitochondrial outer membrane disruption and cytochrome c release, which in turn activates caspase-3 [14]. Vitamin D (VD) treatment significantly improved MTX-induced changes in caspase-3 expression, revealing its anti-apoptotic action [7].

The emerging evidence has demonstrated that an increase in oxidative stress and intracellular ROS is caused by activation of TRPM2 channel [3,4]. Excessive  $Ca^{2+}$  influx to the cytosol via activation of TRPM2 causes cellular damage although its inhibition prevents cell death [19]. Studies have reported that antioxidants decrease oxidative stress, consequently leading to a reduction in TRPM2 activation [4,20]. In MTX group TRPM2 expression was significantly higher than that of other groups. The reason for this increase may be MTX-induced oxidative stress, or it may be the response of cardiac tissue to MTX toxicity via TRPM2 ion channel activation. Vitamin D (VD) significantly inhibited TRPM2 expression in MTX+VD group by reducing oxidative stress, as ROS inhibition leads to reduced TRPM2 activation [20]. Thus, VD which inhibits ROS, may also affect TRPM2 channel activity, consistent with earlier findings [6].

Elevated levels of ROS, which have a significant impact on pathogenesis of cardiotoxicity, lead to the irreversible damage of the cell's DNA, proteins, lipids, and membranes [15]. The MTX decreases antioxidant defense system's effectiveness, which protects cells from cardiac oxidative stress [18]. Similarly, MTX group had much lower cardiac GSH-Px activity than control group. This result was consistent with previous studies that reported decreased GSH-Px, following MTX administration [8,18]. Malondialdehyde (MDA), which is the by-product of lipid peroxidation has been characterized as both an indicator of tissue damage and a marker of oxidative stress induced by MTX [1,18]. Also, MTX caused a significant increase in MDA levels

in rat cardiac tissue, as a result of tissue damage caused by binding lipids in cell membranes [1,8,18]. In contrast to MTX group, VD treatment reduced tissue MDA levels, albeit non-significantly, and increased GSH-Px activity.

## CONCLUSION

The present study has revealed that administering a single dose of VD 200 IU/day for 21 days prevents myocardial toxicity in rats. Also, MTX-induced cardiac damage might be caused by oxidative stress and VD improves oxidative stress by reversing cytotoxicity and, in particular, cardiac tissue damage caused by MTX. Supplementation with VD may be a promising therapeutic approach to protecting cardiac tissue from chemotherapeutic toxicities, particularly those caused by MTX therapy. Moreover, the increased TRPM2 immunoreactivity in MTX group may potentially contribute to pathophysiology of cardiotoxicity, and thus, should be investigated as a means of preventing chemotherapy-induced cytotoxicity.

## DECLARATIONS

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### *Ethical approval*

This study was approved by the Animal Research Ethics of Kahramanmaraş Sutcu Imam University (approval no. 2022/04-02).

### *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### *Conflict of Interest*

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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