Original Paper

Effects of 1, 25-Dihydroxyvitamin D3 on Experimental Autoimmune Myocarditis in Mice

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Key Words

Experimental autoimmune myocarditis • 1, 25(OH)₂ D₃ • Apoptosis • Autophagy

Abstract

Background/Aims: Myocarditis is an important inflammatory disease of the heart which causes life-threatening conditions. 1, 25(OH)₂ D₃ has effects on multiple systems and diseases. The present study was aimed to investigate the effect of 1, 25(OH)₂ D₃ on experimental autoimmune myocarditis (EAM), and explored the underlying mechanisms involved. **Methods:** EAM was induced by immunizing BALB/c mice with cardiac α -myosin heavy chain peptides (MyHC- α). 1, 25(OH)₂ D₃ (1,000 ng/kg once) or vehicle was administered intraperitoneally every other day during the entire experiment. On day 21, transthoracic echocardiography was performed and cardiac inflammatory infiltration was detected by hematoxylin and eosin (HE). The terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay, and Western blots for the expression of protein caspase-3 and cleaved-caspase3 were used to evaluate apoptosis. Transmission electron microscopy and Western blots for the expression of protein Beclin-1, LC3B, and P62 were used to evaluate autophagy. **Results:** The ratio of heart weight/body weight was significantly reduced in 1, 25(OH)₂ D₃ -treated EAM mice, compared with vehicle -treated ones. 1, 25(OH)₂ D₃ treatment improved cardiac function, diminished cell infiltration in cardiac, suppressed myocardial apoptosis, decreased the number of autophagosomes, and decreased the protein expression of Beclin-1, LC3-II and p62. **Conclusions:** The present results demonstrated that administration of 1, 25(OH)₂ D₃ decreased EAM severity. 1, 25(OH)₂ D₃ treatment may be a feasible therapeutic approach for EAM.

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Introduction

Myocarditis, defined as inflammation of the heart with severe ventricular dysfunction, is a major cause of dilated cardiomyopathy and heart failure in individuals younger than 40 years old, which often leads to life-threatening arrhythmia and cardiac sudden death [1]. The pathogenesis of myocarditis remains undefined. Elevated circulating autoantibodies are commonly found in patients with myocarditis or dilated cardiomyopathy, and autoantibody removal improves cardiac function [2, 3]. Autoimmunity is considered to play an important role in the pathogenesis of myocarditis, and cardiac myosin is one of the main autoantigens in virus-induced myocarditis in mice [4]. Many attempts have been made to find potential therapeutic targets for myocarditis, but no specific and effective treatment strategy has been established [1, 5].

Autophagy, a conserved intracellular bulk degradation mechanism, is an adaptive response to harsh conditions, allowing cells to recycle damaged organelles and misfolded cellular proteins. The core of this process is the formation of autophagosomes. Autophagosomes, which have a double membrane, have nonselective cytoplasmic components, such as mitochondria and peroxisomes that are often transported to the lysosome/vacuole for degradation [6]. Autophagy can be induced under various stresses, such as starvation and infection, and it can provide nutrients and energy under starvation conditions [7]. Modest autophagy plays an important role in maintaining general homeostasis and participating in various physiological and pathological conditions, such as cell development, cancer, and pathogenic infections [8-10].

Vitamin D is a fat-soluble secosteroid hormone that is obtained from dietary sources or from endogenous production in the skin following exposure to adequate sunlight [11]. 1, 25-Dihydroxyvitamin D₃ (1, 25(OH)₂ D₃) is the active form of vitamin D. It exerts its effects by binding to the vitamin D receptor (VDR), which is a ligand-modulated nuclear receptor transcription factor [12, 13]. 1, 25(OH)₂ D₃ has high receptor affinity and mediates most biological effects of vitamin D. It exerts pleiotropic effects on multiple systems and diseases, including calcium and bone homeostasis, the regulation of innate and adaptive immune responses, and the modulation of cancer growth and development, cell proliferation, apoptosis, and many chronic disorders [12, 14].

Vitamin D deficiency is associated with increased risk of colon, lung, ovarian, prostate, bladder and breast cancer, and poor prognosis of hematological malignancies [15-18]. Low vitamin D levels are associated with increased incidence of autoimmune disorders, such as systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA) and type 1 diabetes [19].

EAM is an animal model for human acute myocarditis. Based on the above findings, in this study, we aimed to investigate the effect of 1, 25(OH)₂ D₃ on an EAM mouse model.

Materials and Methods

Animals

Male BALB/c mice aged 6 – 8 weeks old were purchased from the Experimental Animal Center of Wuhan University (Hubei province, China). All of the animals were housed in a specific pathogen-free mouse room in the experimental animal center of Tongji Medical College. They had free access to standard rodent chow and water. All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No.85-23, Revised 1996) and were approved by the Animal Care and Use Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Induction of EAM and groups

Eight-week-old male BALB/c mice were subcutaneously injected with cardiac α -myosin heavy chain peptides (MyHC- α , sequence: acetyl-SLKLMATLFSTYAS; purity > 95%; GL Biochem, Shanghai, China),

dissolved in phosphate-buffered saline (PBS) (2 mg/ml), and emulsified 1:1 with complete Freund's adjuvant (CFA; Sigma-Aldrich, America). Each mouse was subcutaneously injected with 200 µl of emulsion (containing 200 µg of MyHC-α peptide) on days 0 and 7 to induce EAM [20]. BALB/c mice were randomly divided into the following three groups: normal control (control group, n = 10), EAM+ vehicle (EAM group, n = 10), and EAM + 1, 25(OH)₂ D₃ (1, 25(OH)₂ D₃ group, n = 10). The control group mice were treated with complete Freund's adjuvant mixed with PBS. The EAM+1, 25(OH)₂ D₃ group was treated with MyHC-α and 1, 25(OH)₂ D₃. 1, 25(OH)₂ D₃ (Sigma Chemical, America) was dissolved in 95% ethyl alcohol solution prepared for stock solution and diluted in peanut oil for intraperitoneal injection. The dosage of 1, 25(OH)₂ D₃ was 1,000 ng/kg in a single injection. The injection was administered every other day throughout the entire experiment, and the first injection was given on the day 0. The EAM+vehicle group was treated with MyHC-α and vehicle, and the mice received vehicle injections according to the same schedule as that used for the EAM+1, 25(OH)₂ D₃ group. The assessment and analysis of acute EAM was conducted on day 21. No mice died during the experiments.

Echocardiography

On day 21, mice were anesthetized with 1.5% isoflurane, and transthoracic echocardiography was performed using a Vevo 1100 Imaging System (VisualSonics, Toronto, Canada) equipped with a 30-MHz transducer (M-mode) according to the methods described in previous reports [4]. Left ventricular internal dimensions at end-systole and end-diastole (LVEDs and LVEDd) were measured. Three consecutive cycles were measured for each assessment, and the average values were recorded. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were then calculated. All echocardiography assessments were performed by the same investigator who was blinded to the treatments.

Tissue harvesting and histopathology

The mice were weighed and euthanized, and the chests were then opened. The hearts were removed and immediately placed in ice-cold PBS. The hearts were weighed, and the ratio of heart weight to body weight (HW/BW) was calculated. Ventricular tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Then, 5-µm sections were cut transversely and stained with hematoxylin and eosin (H&E). The severity of myocarditis was assessed based on the infiltrated area ratio, which was the percentage of the heart section with inflammatory infiltrates compared with the whole transverse heart section, and the histological pathological scoring system was as follows: grade 0, no infiltration; grade 1, infiltrated area ratio less than 25%; grade 2, infiltration area of 25% to 50%; grade 3, infiltration area of 50% to 75%; and grade 4, infiltration area more than 75% [21, 22]. Five sections from each heart were scored by two independent researchers who were blinded to the treatments.

TUNEL assay

Apoptotic cells in the myocardium were identified by terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) according to the manufacturer's instructions (Roche Diagnostics, Germany). Apoptotic nuclei were detected by green fluorescein staining, total nuclei were identified by DAPI labeling (blue), and cardiomyocytes were identified by anti-α-actin antibody staining (red).

Transmission electron microscopy

Autophagosomes were detected as previously described [23]. Briefly, heart tissues were fixed in 3% glutaraldehyde for 24 h and 1% osmium tetroxide for 2 h. The ultrathin sections were stained with uranyl acetate and lead citrate. Random sections were assessed by an electron microscopy technician who was blinded to the treatments. For each section, 20 electron micrographs were obtained with magnifications of 1,700 and 3,500 x.

Western blot analysis

Total proteins in the heart tissues were prepared using standard protocols, and the protein concentrations in the lysates were determined with a BCA Protein Assay Kit (Thermo Scientific). Equal amounts of protein (80 µg/lane) were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in Tris-buffered saline with Tween 20 (TBST) containing 5% skim milk for 2

h at room temperature, and they were then incubated with primary antibodies against Beclin-1 (1:1,000, CST), LC3B (1:500, CST), caspase 3 (1:1,000, CST), GAPDH (1:3,000, CST), and P62(SQSTM1/sequestome 1) (1:1,000, CST) at 4°C overnight, followed by washing with TBST. The samples were then incubated with corresponding HRP-conjugated secondary antibodies (1:4,000, Antgene Biotechnology, China) for 2 h at room temperature. The expression levels of target protein bands were detected using enhanced chemiluminescence (Millipore) and were quantified by densitometry with the BioRad image analysis program Image Lab Software.

Statistical analysis

Data are presented as the means ± SEMs and were analyzed using SPSS 18.0 for Windows. Statistical analysis was performed with one-way analysis of variance (ANOVA) when appropriate. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn's post hoc test. $P < 0.05$ was considered statistically significant.

Results

1, 25(OH)₂ D₃ improved cardiac function of EAM mice

As shown in Fig. 1A–E, the LVEF and LVFS were significantly reduced in the EAM group compared with the normal control group (LVEF $73.40\% \pm 0.96\%$ and LVFS $41.10\% \pm 0.76\%$ in the normal control group vs. LVEF $47.05\% \pm 1.77\%$ and LVFS $23.25\% \pm 1.1\%$ in the EAM group, $P < 0.01$). Compared with the EAM group, 1, 25(OH)₂ D₃ treatment improved LVEF ($62.71\% \pm 1.68\%$ vs. $47.05\% \pm 1.77\%$, $P < 0.01$) and LVFS ($32.98 \pm 1.14\%$ vs. $23.25 \pm 1.1\%$, $P < 0.01$). Similarly, LVEDd and LVEDs were increased in the EAM group compared with the normal control group (LVEDd 3.16 ± 0.26 in the normal control group vs. LVEDd 3.94 ± 0.14

Fig. 1. Echocardiographic analyses. Mice were induced with EAM, and they were treated with 1, 25(OH)₂ D₃ (VitD3) or vehicle. Cardiac function was determined by echocardiography on day 21. (A) Representative images of M-mode echocardiograms; (B) LVEF(%); (C) LVFS (%); (D) LVEDd (mm); (E) LVEDs (mm). Values are expressed as the means ± SEMs, $n = 6$ animals per group. * $P < 0.05$ or ** $P < 0.01$ vs. the control group; # $P < 0.05$ or ## $P < 0.01$ vs. the EAM group. VitD3 treatment significantly prevented the progression of left ventricular dysfunction.

Fig. 2. Inflammatory infiltration was reduced by 1, 25(OH)₂ D₃ treatment. Heart tissue sections were stained with hematoxylin and eosin (H&E) to evaluate inflammation. (A) Representative images of tissues stained with H&E (magnification $\times 200$), Scale bar = 100 μ m; (B) The pathological scores of heart sections; (C) The ratios of HW/BW. Values are expressed as the means $\pm$ SEMs, $n = 10$ animals per group. * $P < 0.05$ or ** $P < 0.01$ vs. the control group; # $P < 0.05$ or ## $P < 0.01$ vs. the EAM group.

in the EAM group, $P < 0.05$; LVEDs 1.68 ± 0.1 in the normal control group vs. LVEDs 3.06 ± 0.1 in the EAM group, $P < 0.01$). Compared with the EAM group, LVEDd and LVEDs were decreased in the 1, 25(OH)₂ D₃ group (LVEDd 3.45 ± 0.14 in the 1, 25(OH)₂ D₃ group vs. LVEDd 3.94 ± 0.14 in the EAM group, $P = 0.082$; LVEDs 2.20 ± 0.11 in the 1, 25(OH)₂ D₃ group vs. LVEDs 3.06 ± 0.1 in the EAM group, $P < 0.01$).

Together, these results showed that left ventricular dysfunction occurred in EAM mice and that 1, 25(OH)₂ D₃ played a role in improving cardiac function in EAM mice.

1, 25(OH)₂ D₃ reduced inflammatory infiltration in EAM mice

As shown in Fig. 2A, significant inflammatory cellular infiltration was observed in the EAM mice, and 1, 25(OH)₂ D₃ ameliorated the extent of inflammatory infiltration. The pathological scores of heart sections in the EAM group were significantly elevated compared with those in the normal control group ($P < 0.01$), and they were much lower in the 1, 25(OH)₂ D₃ -treated EAM group compared with the vehicle -treated EAM group ($P < 0.05$; Fig. 2B). The ratio of HW/BW was increased in the EAM group compared with the control group ($P < 0.01$), and it was much lower in the 1, 25(OH)₂ D₃ -treated EAM group compared with the vehicle -treated ones ($P < 0.05$; Fig. 2C).

Administration of 1, 25(OH)₂ D₃ reduced myocardiocytes apoptosis in EAM mice

To determine the effect of 1, 25(OH)₂ D₃ on myocardiocytes apoptosis, the EAM mice were treated with vehicle or 1, 25(OH)₂ D₃, and myocardiocytes apoptosis was assessed. Our results showed that, compared with vehicle treatment, the administration of 1, 25(OH)₂ D₃ markedly reduced EAM- induced myocardiocytes apoptotic responses as assessed by reduced TUNEL staining ($P < 0.01$; Fig. 3A). In addition, we examined the protein expression of caspase-3 and cleaved-caspase-3. In the EAM group, the expression of caspase-3 and cleaved-caspase-3 were increased compared with those in the control group ($P < 0.01$).

Fig. 3. 1, 25(OH)₂ D₃ treatment reduced cardiomyocytes apoptosis in EAM mice. (A) Myocardocytes apoptosis was determined by TUNEL (green, apoptotic nuclei; blue, DAPI, total nuclei; red, α -actin, cardiomyocytes), Scale bar = 50 μ m. (B) Representative Western blot and quantitative analysis of pro-caspase-3, and cleaved-caspase-3. Values are expressed as the means $\pm$ SEMs, n = 6 animals per group. *P < 0.05 or **P < 0.01 vs. the control group; #P < 0.05 or ##P < 0.01 vs. the EAM group.

1, 25(OH)₂ D₃ treatment decreased the expression of caspase-3 and cleaved-caspase-3 (P < 0.01; Fig. 3B).

Overall, these results showed that 1, 25(OH)₂ D₃ suppressed cardiomyocytes apoptosis in EAM mice.

Administration of 1, 25(OH)₂ D₃ influenced autophagy in EAM mice

In addition to apoptosis, we assessed the effect of 1, 25(OH)₂ D₃ on autophagy. As shown in Fig. 4A, transmission electron microscopy (TEM) revealed increased autophagosomes in the vehicle-treated EAM mouse hearts, while 1, 25(OH)₂ D₃ treatment reduced the number of autophagosomes. Due to their importance in autophagosome formation, we measured the expression of LC3-II (microtubule-associated protein 1 light chain 3-II), the expression of Beclin-1, and the expression of SQSTM1/p62. P62 is a marker of autophagy-mediated protein degradation, and its level is a marker of autophagic flux [24, 25]. As shown in Fig. 4B and 4C, Western blot analysis showed that the protein expression of Beclin-1 and LC3-II were significantly increased in the EAM hearts (P < 0.05). The expression of p62 was not reduced and was even increased in the EAM group (Fig. 4D). Additionally, the LC3-II level was unchanged in chloroquine (CQ, lysosome inhibitor that prevents autophagosome-lysosome fusion)-treated EAM mice compared with vehicle-treated EAM mice (Fig. 4E). These results suggested that EAM induced autophagosome formation with abnormal autophagosome

Fig. 4. 1, 25(OH)₂ D₃ treatment influenced autophagy in EAM mice. (A) Representative transmission electron microscopy images and the results of the statistical analysis of the numbers of autophagosomes from 20 fields (n = 3 hearts per group). Representative Western blot and quantitative analysis of Beclin-1 (B), LC3-II (C), and p62 (D). An LC3 turnover assay was conducted in our subsequent experiment (E) and showed that the LC3-II levels remained unchanged in the chloroquine-treated (2mg/Kg intraperitoneally injected 2 h before sacrifice) EAM mice compared with the vehicle-treated EAM mice. Values are expressed as the means ± SEMs, n = 6 hearts per group. *P < 0.05 or **P < 0.01 vs. the control group; #P < 0.05 or ##P < 0.01 vs. the EAM group.

clearance. Compared with the EAM group, 1, 25(OH)₂ D₃ treatment reduced the expression of Beclin-1, LC3-II, and p62 protein (Fig. 4B–D; P < 0.05). Taken together, these results suggested that 1, 25(OH)₂ D₃ modulated autophagy dysfunction in EAM mice.

Discussion

Myocarditis is an acute inflammatory disease of the heart, characterized by inflammatory cellular infiltrations. Studies have suggested that immune responses, inflammatory cytokines, oxidative stress, endoplasmic reticulum (ER) stress, and cell apoptosis are involved in the pathogenesis and development of EAM [26–28]. In this study, our data demonstrated that 1, 25(OH)₂ D₃ treatment can ameliorate EAM severity by reducing cardiac inflammatory infiltration and suppressing myocardial apoptosis. Fewer inflammatory cells in the cardiac

tissue of the 1, 25(OH)₂ D₃ group were observed compared with the EAM group. The pathological scores for the 1, 25(OH)₂ D₃ treated EAM mice were lower than those for the vehicle treated EAM mice. 1, 25(OH)₂ D₃ treatment attenuated the activation of caspase-3. The TUNEL staining results showed that the number of apoptotic cells was reduced by 1, 25(OH)₂ D₃ treatment. Echocardiographic results suggested that treatment with 1, 25(OH)₂ D₃ improved cardiac function. These data revealed an important role for 1, 25(OH)₂ D₃ in improving cardiac function accompanied with myocardiocytes apoptosis reduction.

Vitamin D has been extensively studied. In addition to bone metabolism and mineral ion homeostasis, vitamin D participates in many biological processes. It can limit inflammation and inhibit cancer development and progression, and it has an anti-inflammatory effect on a variety of cell types, such as endothelial cells and immune cells [29-32]. 1, 25(OH)₂ D₃ can exert effects on T cells and B cells directly. It can inhibit Th1 cell development and enhance the Th2 cells development, reducing the production of proinflammatory cytokine [19]. What's more, it can induce B cells apoptosis, inhibiting plasma-cell differentiation and autoantibody production [19]. The possible effects of 1, 25(OH)₂ D₃ on lymphocytes and immunomodulatory may contribute to the improvement of cardiac function in EAM.

Vitamin D deficiency is prevalent in adults because of inadequate intake from food or decreased exposure to sunlight and is important in the pathology of many types of diseases. Low vitamin D levels have been associated with an increased risk for breast, colorectal, and prostate cancer [33, 34], as well as autoimmune disorders and periodontal disease [35, 36]. In the cardiovascular system, epidemiological evidence suggests a correlation between low vitamin D status and a high incidence of cardiovascular diseases [37]. Vitamin D deficiency is associated with elevated blood pressure, electrolyte imbalances, renin-angiotensin system (RAS) activation, ventricular hypertrophy and heart failure [37, 38].

Autophagy, a lysosome-mediated catabolic pathway, has an important role in maintaining cellular homeostasis and occurs in almost all eukaryotic cells [39]. Defects in autophagy may be associated with cancer, neurodegenerative diseases, and aging [8]. Autophagy is considered to have a pro-survival role because the deletion of some critical genes for autophagy accelerates rather than inhibits cell death [40-42]. However, some evidences showed that unbridled autophagy may result in cell death by degrading some critical proteins or excessive self-digestion [43-46]. Impaired autophagosome clearance may lead to ischemia/reperfusion-induced cardiomyocyte cell death [47]. Wong et al. [48] reported that CVB3 infection induced the activation of autophagy machinery in the host, which could enhance viral replication. Yuan et al. [49] showed that autophagy contributed to IL-17-induced autoantibody production in plasma cells in EAM and inhibiting autophagy with 3-MA (autophagy inhibitor) alleviated the effect of rIL-17. In this study, our results showed that increased autophagosome formation was observed in EAM mice, as evidenced by the increased number of autophagosomes, upregulation of Beclin-1 and LC3-II. An impaired autophagic flux was observed in the EAM mice, as the expression of LC3-II was increased in the EAM mice and the LC3-II level remained unchanged in the chloroquine-treated EAM mice compared with the vehicle-treated EAM mice, the expression of protein P62 was not reduced and was even increased in EAM mice. These results suggested that autophagy dysfunction existed in EAM, as evidenced by an increase in autophagosome formation and abnormal autophagosome degradation by lysosomes.

A growing number of studies have suggested that vitamin D could regulate autophagy at different levels. It can induce autophagy to maintain cell viability, reduce cell apoptosis, and attenuate the severity of disease. Meanwhile, it plays essential roles in regulating autophagy to avoid autophagy dysfunction-mediated cell death. Yao et al. [50] showed that vitamin D inhibited autophagy dysfunction-mediated cell death and improved cardiac function. In the present study, we showed that 1, 25(OH)₂ D₃ treatment improved autophagy dysfunction since the reduction of Beclin-1, LC3-II and p62 protein expression were observed in the 1, 25(OH)₂ D₃ treated EAM mice.

In summary, the present study demonstrated that 1, 25(OH)₂ D₃ is capable of improving cardiac function by inhibiting cardiac inflammatory infiltration, reducing myocardiocytes

apoptosis, and autophagy modulation. 1, 25(OH)₂ D₃ treatment may therefore be a promising therapeutic strategy for the clinical treatment of myocarditis. However, since excessive vitamin D could lead to calcification, further studies are needed to determine the proper dosages and to evaluate the clinical usefulness and effectiveness of treatment for myocarditis in humans.

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Disclosure Statement

The authors declare that they have no interests that could influence the publication of this paper.

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