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# Vitamin D Deficiency Harms Patients with Coronary Heart Disease by Enhancing Inflammation

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**Background:** Vitamin D (VD) deficiency and local inflammation of plaque are potential new risk factors and prevention goals for coronary heart disease (CHD).





**Material/Methods:** This study included 135 CHD patients and 45 chest tightness or chest pain patients (control group). Basic clinical data and serum 25-OH-VD, TNF- $\alpha$ , IL-6, IL-8, and IL-1 $\beta$  of the 2 groups were compared by SPSS 25.0. A CHD rat model was used to explore the potential molecular mechanisms.

**Results:** The serum 25-OH-VD level in the control group was significantly higher compared to the CHD group, and decreased with the worsening of the CHD condition. Logistic regression found that serum 25-OH-VD was a protective factor in the occurrence of CHD. In CHD patients, the level of serum 25-OH-VD had a negative correlation with serum TNF- $\alpha$  ( $r=-0.651$ ,  $P<0.001$ ), IL-6 ( $r=-0.457$ ,  $P<0.001$ ), IL-8 ( $r=-0.755$ ,  $P<0.001$ ), and IL-1 $\beta$  ( $r=-0.628$ ,  $P<0.001$ ). In animal experiments, VD deficiency enhanced the level of serum TC, TG, and LDL-C. VD deficiency could increase the inflammatory response by upregulating the expression of p65 protein and reducing SIRT1 protein expression in heart tissue, thereby inducing or aggravating the state of CHD.

**Conclusions:** Serum 25-OH-VD was a protective factor in the occurrence of CHD, and VD deficiency could induce or aggravate the state of CHD by enhancing inflammation through the NF- $\kappa$ B pathway.

**MeSH Keywords:** **Coronary Disease • Inflammation • Vitamin D**

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## Background

Inflammation plays an important role in the formation of atherosclerosis plaques and the progression of lesions [1,2], which were 2 important processes in the formation of coronary heart disease (CHD). At present, the prognosis of patients with CHD has significantly improved, but CHD still has the highest prevalence rate of all diseases in the world [3]. Some potential new risk factors and prevention goals have attracted widespread attention, such as vitamin D (VD) deficiency [4,5], and local inflammation of plaque [6]. Previous studies [6] have shown that the development of CHD involves 3 interrelated pathophysiological processes: atherosclerosis (AS) formation, plaque rupture, and coronary artery spasm are prone to occur in coronary arteries with AS and cause angina or even myocardial infarction. A variety of inflammatory cytokines played important roles in these 3 pathophysiological processes of CHD.

In recent years, a large number of studies have shown that VD can play an extensive role in the body, including regulating inflammation and immune response, affecting cognitive function, and providing protection against metabolic syndrome and cardiovascular disease [7]. A large number of clinical and epidemiological studies have shown that VD deficiency is closely related to the incidence and mortality of CHD [5,8], but the specific mechanisms have not yet been fully defined [9,10]. It is currently considered that VD might be involved in the pathogenesis of CHD and the progression of the disease through negative regulation of RAS to lower blood pressure, promotion of insulin secretion, improvement in insulin resistance, regulation of cell differentiation and proliferation processes, and anti-inflammatory and anti-atherosclerosis effects [11]. In the present study, we compared the serum 25-OH-VD, TNF- $\alpha$ , IL-6, IL-8, and IL-1 $\beta$ , of the 2 groups and explored the molecular mechanism in a CHD rat model, so as to confirm the relationship between VD deficiency and CHD.

## Material and Methods

### Ethics statement

The present study was performed with the approval of the Ethics Committee of Cangzhou Central Hospital. All aspects of the study complied with the Declaration of Helsinki. In addition, all participants signed the informed consent.

### Patients

There were 180 patients treated in the Department of Cardiology at Cangzhou Central Hospital from May 2015 to May 2017 who had coronary angiography because of chest tightness and chest pain: 145 male patients and 35 female

patients; age  $56.78 \pm 6.95$  years old. They were divided into a CHD group and a control group according to coronary angiography results. There were 135 patients in the CHD group, 111 male patients and 24 female patients (age  $57.23 \pm 6.72$  years old), and the diagnostic standard was that coronary angiography showed at least 1 vessel diameter stenosis  $\geq 50\%$  in left anterior descending (LAD), left circumflex (LCX), or right coronary artery (RA). There were 45 patients in the control group, 34 male patients and 11 female patients (age  $55.43 \pm 7.46$  years old), and their coronary angiography showed normal or major coronary branch narrow diameter  $50\%$ .

Patients who met the following criteria were excluded from this study: 1) Infection with severe infectious diseases, acute heart failure, primary cardiomyopathy, myocarditis, and severe cardiac insufficiency (NYHA class III to IV); 2) previous history of coronary artery disease with stenting or coronary artery bypass grafting; 3) history of acute and chronic hemorrhage with hematologic disorders; 4) chronic liver and kidney insufficiency, connective tissue; 5) drugs that affect glucocorticoids, statins, and anti-epileptic drugs that affect VD levels in the body within the past 3 months; 6) taking VD preparation for a long-term or within the last 3 months.

### Coronary angiography

Coronary angiography was performed according to Judkin's method; briefly, after local anesthesia, the radial artery or femoral artery was used as the puncture path. Left and right coronary angiography were performed respectively. At least 2 positions were projected, and CAG images were recorded. The catheter was used as a reference to determine target vessels and target lesions. The angiography results were analyzed and judged by 2 senior experts without knowing the patient's 25-OH-VD level in terms of site, type, and degree of stenosis.

### Clinical parameters

We recorded the sex and age of each patient. We asked about smoking behavior and family history of hypertension. The height and weight of all participants was measured, and the body mass index ( $\text{kg}/\text{m}^2$ ) was calculated. The self-rating depression scale (SDS) was used to assess patient's mental state. All patients were evaluated after chest pain and chest tightness relief and the patient was ready for discharge.

Each participant was allowed to rest for at least 10 minutes before systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by an automated blood pressure monitor (EW3106, Panasonic, Japan), a repeated measurement was performed on a different day (2-week time interval at least), and the average of the 2 measurements was calculated [12].

## Blood samples

Peripheral blood (5–10 mL) was collected in EDTA-containing tubes, then centrifuge (1000 g) for 10 minutes (5810R, Eppendorf AG, Germany) to collect plasma. The plasma levels of total cholesterol, triglycerides, high-density cholesterol, and low-density cholesterol were measured by an automatic biochemical analyzer (AU680, Beckman Coulter, USA) [13].

## Animal experiment

For the rat model, 3- to 4-week old Sprague Dawley rats were fed for 1 week to adapt to the experimental environment ( $24\pm 1^\circ\text{C}$ , humidity of  $50\pm 10\%$ , natural lighting conditions, and ensuring adequate food and drinking water), then the rats were randomly divided into a control group and a VD-deficient group. The control group rats were given normal feed and fed under normal conditions. VD-deficient rats were fed with VD deficient feed under conditions of yellow light (without UV-illumination). Blood samples were taken by posterior eyeball venous plexus blood sampling; serum VD content was measured at 4 weeks, 6 weeks, and 8 weeks.

After 8 weeks of feeding, the control rats were randomly divided into a healthy group ( $n=10$ ) and a CHD group ( $n=10$ ). VD-deficient rats were randomly divided into VD-CHD group ( $n=10$ ) and VD poor-CHD group ( $n=10$ ). Rats in the Healthy group were fed with normal basal diet and normal light conditions. Rats in CHD group and VD-CHD group were fed with high-fat diet and normal light conditions. Rats in VD poor-CHD group were given high-fat diet and had yellow light (without UV-illumination) conditions.

After 8 weeks of feeding, eye fundus blood samples were taken at 10, 12, 14, and 16 weeks. At 16 weeks, the rats were anesthetized with 350 mg/kg of 4% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Then we cut into the chest to expose the heart, inserted the injection needle into the left ventricle, cut the right atrial appendage, rapidly perfused with the normal saline (100 mL), and quickly removed the heart, fixing the heart in 10% formalin, followed by dehydrated. At the distance from the ascending aorta, at a 0.5 cm apex, the heart was transected into 3 segments, and the tissue blocks were taken and embedded in paraffin. The thickness was 4  $\mu\text{m}$ . The sections were stained with hematoxylin and eosin (H&E).

## ELISA assay

For ELISA assay we used human vitamin D assay ELISA Kit (AC-57F1, IDS, UK) for detection of human or rat serum VD content; human IL-10 assay ELISA Kit (50R-E.1095H, BioValue, AU) for human or rat IL-10; human IL-8 assay ELISA Kit (H-EL-IL-8,

**Table 1.** RT-qPCR primer sequence.

Name	Sequence (5'-3')
TNF- $\alpha$	F: CAGGCGGTGCCTATGTCTC
	R: CGATCACCCCGAAGTTCAGTAG
IL-6	F: CTGCAAGAGACTTCCATCCAG
	R: AGTGGTATAGACAGGTCTGTTGG
IL-8	F: TCGAGACCATTACTGCAACAG
	R: CATTGCCGGTGGAAATTCCTT
IL-1 $\beta$	F: CTTACTGACTGGCATGAGGATCA
	R: GCAGCTCTAGGAGCATGTGG
GAPDH	F: GATGAACCTAAGCTGGGACCC
	R: TGTGAACGGATTGGCCGTA

ZYscience, USA) for human IL-8; human IL-6 assay ELISA Kit (H-EL-IL-6, ZYscience, USA) for human or rat IL-6; human TNF- $\alpha$  assay ELISA Kit (50R-E.1693H, BioValue, AU) for human TNF- $\alpha$ .

## Real-time fluorescence quantitative PCR

TRIzol was used to extract the total RNA of the heart tissue of rats. The extracted RNA was reverse transcribed into cDNA by using PrimeScript™ RT Master Mix Reverse Transcription kit (RR036B, Takara, Beijing, China). PCR parameters were:  $37^\circ\text{C}$  for 60 minutes,  $85^\circ\text{C}$  for 5 seconds, then 20  $\mu\text{L}$  real-time fluorescence quantitative PCR (RT-qPCR) system was prepared according to the SYBR Green qPCR Master Mix kit instructions (638320, TakaRa, Beijing, China) and amplified using ABI 7500 fluorescence quantitative PCR instrument (Applied Biosystems, Maryland, USA). PCR parameters were:  $95^\circ\text{C}$  for 30 seconds, and then  $90^\circ\text{C}$  for 5 seconds and  $65^\circ\text{C}$  for 30 seconds, for 40 cycles. The details of RT-qPCR primer sequence are shown in Table 1.

## Western blot

Heart tissue of rat lysates were separated by SDS-page and then transferred to PVDF membranes. Primary antibody was selected as follows: anti-SIRT1 (ab110304, 1: 5000, Abcam, UK) or anti-p65 (ab16502, 1: 1000, Abcam, UK), or anti-GAPDH (ab9484, 1: 3000, Abcam, UK). And second antibody was selected as follows: goat anti-rabbit (ab150077, 1: 1000). Primary antibody was incubated overnight at  $4^\circ\text{C}$  and second antibody was incubated for 1 hour at room temperature.

## Statistical analysis

Data are presented as mean  $\pm$  standard deviation and were analyzed by SPSS 25.0. Student's *t*-test or chi-square test

**Table 2.** Basic clinical data in the control group and the CHD group.

Variables	Control (n=45)	CHD (n=135)	t or $\chi^2$	P value
Age (n/%)	55.43±7.46	57.23±6.72	1.324	0.281
Male (n/%)	34 (75.6)	111 (82.2)	0.958	0.328
Hypertension (n/%)	14 (31.1)	70 (51.9)	5.833	0.016
Diabetes (n/%)	4 (8.9)	62 (45.9)	19.936	0.000
High cholesterol (n/%)	16 (35.6)	90 (66.7)	13.499	0.000
Smoking (n/%)	12 (25.7)	65 (48.1)	6.362	0.012
SBP (mmHg)	136.42±25.68	141.47±21.52	1.425	0.182
DBP (mmHg)	82.62±17.47	86.65±13.54	1.593	0.152
BMI (kg/m <sup>2</sup> )	24.43±3.25	25.71±3.62	2.845	0.005
TC (mmol/L)	4.62±0.82	5.13±0.94	2.982	0.004
TG (mmol/L)	1.54±0.81	2.15±0.15	7.182	0.000
LDL-C (mmol/L)	2.55±0.56	3.29±0.78	8.294	0.000
HDL-C (mmol/L)	1.59±0.32	1.48±0.39	2.514	0.009
SDS	39.69±6.59	50.68±8.94	22.354	0.000

was used to compare differences between 2 groups. One-way ANOVA with Duncan's post-hoc test was used for comparing multiple groups. The correlation between serum level of 25-OH-VD and inflammatory factors in patients with CHD was analyzed by Pearson's correlation coefficient. Logistic regression models were constructed to determine the odds ratio (OR) and 95% confidence interval (CI) for putative risk factors associated with CHD.  $P < 0.05$  was considered a statistically significant difference.

## Results

### Basic clinical data

The basic clinical data of the CHD group (n=135) and the control group (n=45) are present in Table 2. There was no significant difference in age, gender, SBP, and DBP, but there was significant difference with hypertension or diabetes or high cholesterol, smoking, BMI, TC, TG, HDL-C, and SDS between the control group and the CHD group.

### Serum level of 25-OH-VD in different patients

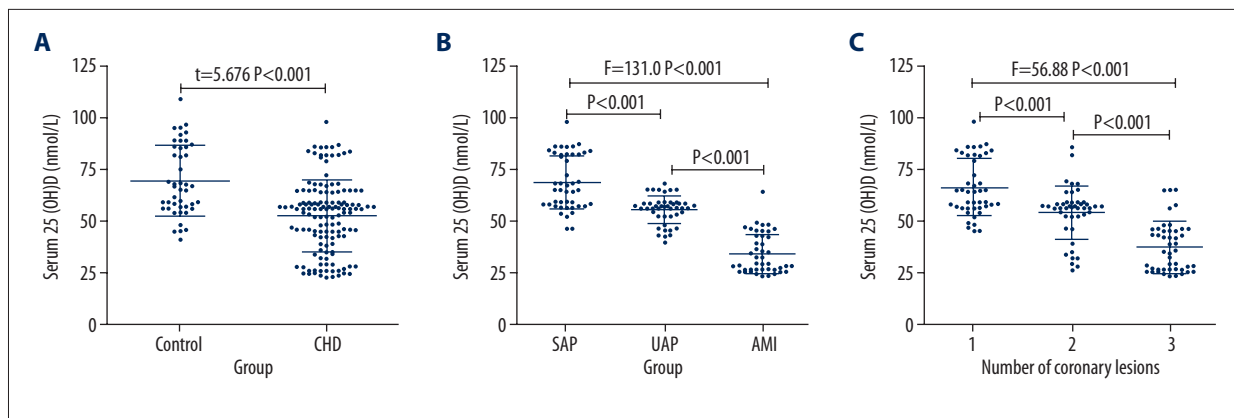
The serum level of 25-OH-VD in the CHD group (n=135) and the control group (n=45) were measured by ELISA kit. As shown in Figure 1A, the serum level of 25-OH-VD in the CHD group was significantly less than that in the control group. According to clinical data and angiography of patients with CHD, 135 CHD patients were divided into a stable angina group (SAP),

an unstable angina group (UAP), and an acute myocardial infarction group (AMI). We found that there were significant differences in serum 25-OH-VD between the 3 groups: serum 25-OH-VD in the SAP group was significantly higher than that in the UAP group, and the UAP group was significantly higher than that in AMI group (Figure 1B). We also found that because the number of coronary lesions in patients with CHD was different, there was also a significant difference in serum 25-OH-VD levels (Figure 1C).

In our logistic regression analysis, we made CHD (1=yes, 0=no) as the dependent variable, age, male (0=female, 1=male), hypertension (0=no, 1=yes), diabetes (0=no, 1=yes), hyperlipidemia (0=none, 1=yes), smoking (0=no, 1=yes), BMI, TC, TG, LDL-C, HDL-C, 25-OH-VD as independent variables. We found that 25-OH-VD was a protective factor in the occurrence of CHD (Table 3).

### Correlation between 25-OH-VD and inflammatory factors in CHD group

Atherosclerosis is the core pathogenesis of CHD, and the inflammatory response plays an important role in the formation of atherosclerosis plaques and the progression of lesions. It is also a key factor that can cause plaque stability to decline and even the rupture of plaque fibrosis caps [1]. In clinically settings, the stability of atherosclerosis plaques is often assessed indirectly by measuring serum inflammatory factors in patients with CHD [2]. In this paper, we analyzed the correlation between serum inflammatory factors and 25-OH-VD in



**Figure 1.** The level of 25-OH-VD in serum. (A) Serum level of 25-OH-VD in the control group and the CHD group; (B) Serum level of 25-OH-VD in the patient subgroups SAP (n=45), UAP (n=45), and AMI (n=45), which were in the CHD group; (C) Serum level of 25-OH-VD in patients in CHD group with 1, 2, and 3 coronary lesion.

**Table 3.** Results of the logistic regression analysis.

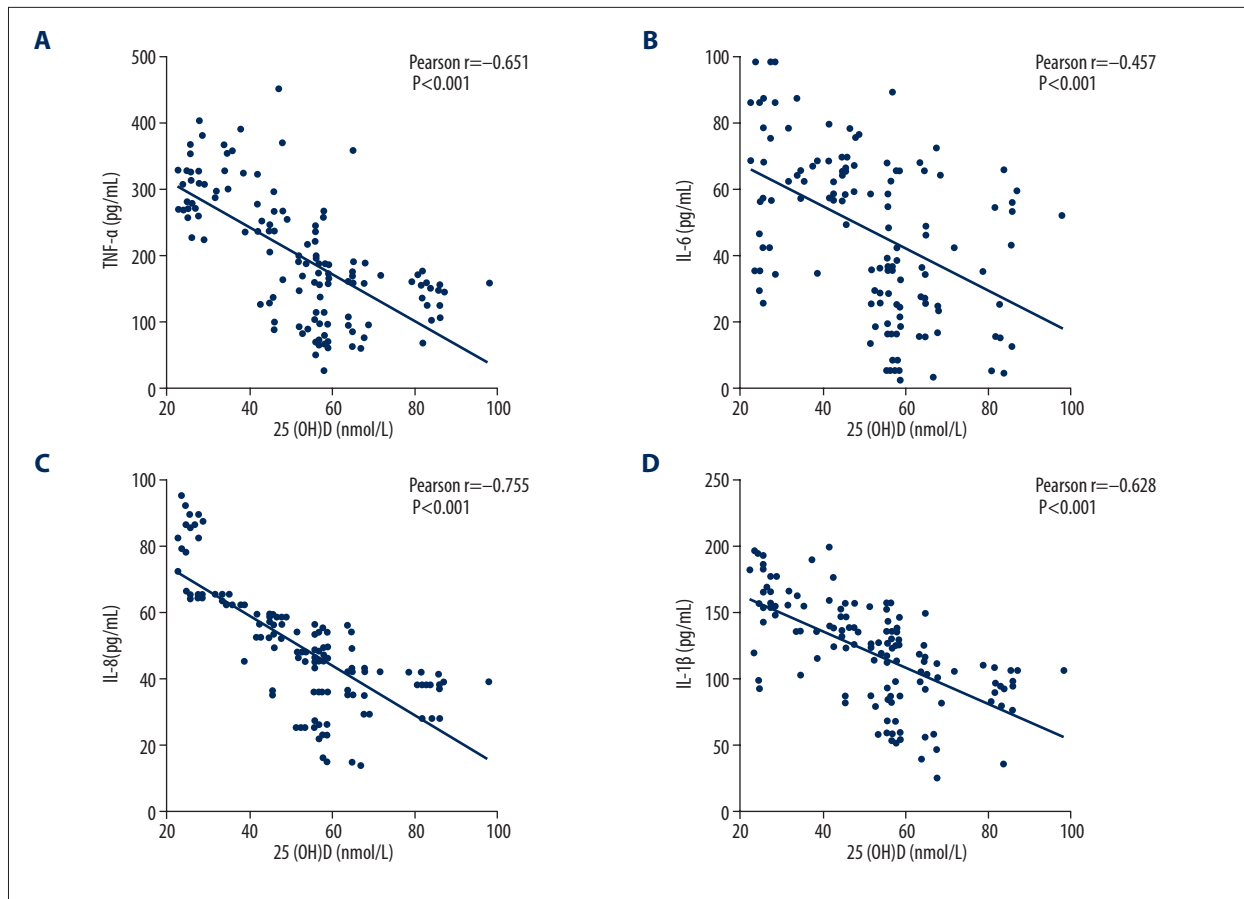
Variables	Sig.	Exp(B)	95%CI
Age	0.628	1.024	0.935–1.112
Male	0.854	0.859	0.182–4.125
Hypertension	0.635	0.756	0.256–2.215
Diabetes	0.008	8.230	1.784–38.214
High cholesterol	0.061	0.338	0.109–1.051
Smoking	0.006	1.112	0.356–3.374
BMI	0.187	1.135	0.942–1.412
TC	0.639	1.125	0.625–2.078
TG	0.628	0.846	0.425–1.674
LDL-C	0.224	1.825	0.694–4.447
HDL-C	0.065	0.218	0.043–0.991
25(OH)D	0.000	0.909	0.869–0.951

CHD patients, and we found that the level of serum 25-OH-VD in CHD patients had a negative correlation with serum TNF- $\alpha$  ( $r=-0.651, P<0.001$ ), IL-6 ( $r=-0.457, P<0.001$ ), IL-8 ( $r=-0.755, P<0.001$ ) and IL-1 $\beta$  ( $r=-0.628, P<0.001$ ). See Figure 2 for details.

**VD deficiency exacerbates High-fat food damage in rats**

First, VD deficiency food was used to feed rats to establish animal models of VD deficiency (Figure 3A). Second, high fat food was used to establish animal models of CHD. It should be noted that 2 rats in VD-CHD group and 4 rats in VD poor-CHD group died during the 12–14 weeks period, and 1 rat in VD-CHD group and 2 rats in VD poor-CHD group died during the 14–16 weeks period.

At 16 weeks, we found that the thickness of the coronary artery was uniform, and no abnormalities were found in the adventitia, media, or intima, and there was no CAA lesion in Healthy group (Figure 3B, upper left). In the VD poor-CHD group, the intima of a coronary artery or the entire lumen of the coronary artery was thickened with uneven thickness, lipid spots, or lipid streaks were visible in the intima, foam cells, and vascular wall fibers were proliferated to varying degrees, and the coronary vessel wall was deformed to form a lipid pool (Figure 3B, lower right). The inner surface of the block was destroyed and covered by a proliferating fibrous cap on the lipid pool. The membrane showed local calcification, and the CAA lesions were obvious. In the CHD group (Figure 3B, upper right) and VD-CHD group (Figure 3B, lower left), the thickness of the coronal lateral wall was slightly thicker than that of the normal group. Lipid spots and bi-directional bulging plaques were seen



**Figure 2.** Correlation between serum level of 25-OH-VD and TNF- $\alpha$ , IL-6, IL-8, and IL-10 in the CHD group.

in the intima. A few fibrous caps were seen on the surface of individual plaques. The local smooth muscle cells in the membrane were disordered and atrophied and thinned, but their CAA lesions were significantly better than those in the model group. At different times, blood sample were obtained from the rat's posterior vein plexus of the eyeball, and the level of serum TC, TG, HDL-C, LDL-C were detected. As shown in Figure 3C–3F, VD deficiency enhanced the level of serum TC, TG, and LDL-C, and reduced the level of serum HDL-C, which was compared with healthy rats at 0–8 weeks and was comparable to CHD rats at 8–16 weeks. However, VD supplementation could reduce this change.

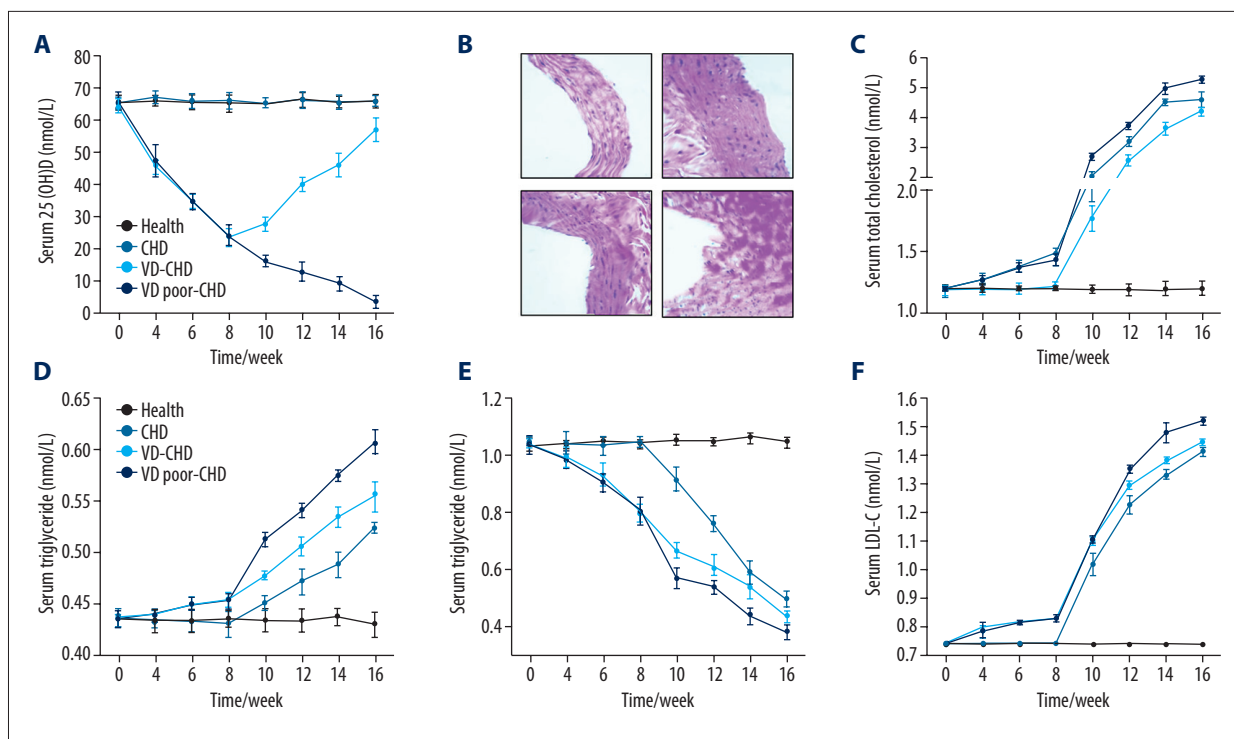
### VD deficiency enhances inflammation in rats

At 16 weeks, all the live rats were sacrificed, and their heart tissue were used to extract RNA and protein. RT-qPCR was used to detect the expression of inflammatory factors mRNA, and we found that VD deficiency enhanced the expression of inflammatory factors mRNA (Figure 4A). In addition, western blot analysis showed that VD deficiency increased the expression of p65 protein and decreased the expression of SIRT1 protein (Figure 4B).

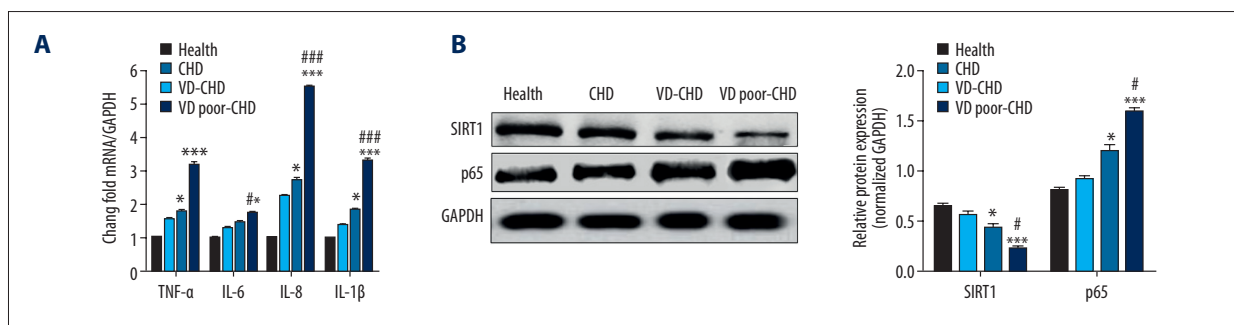
### Discussion

More and more evidence show that in addition to the traditional cardiovascular risk factors, some newly discovered factors are also closely related to CHD, such as VD deficiency and hyperhomocysteinemia. The synthesis in the skin is the main source of physiological conditions [14]. Recent studies have found that VD is essential for human health, such as negative control of RAS to lower blood pressure, increased insulin secretion, improved insulin resistance, regulation of tissue cell differentiation and proliferation process, and has anti-inflammatory, anti-atherosclerosis, and other effects [15].

This study found that the serum 25-OH-VD level in the control group was significantly higher than in the CHD group, and its level decreased with the worsening of the CHD condition. And logistic regression analysis found that 25-OH-VD was a protective factor in the occurrence of CHD. Atherosclerosis is the core of pathological changes in CHD. Early studies have shown that VD deficiency is associated with coronary atherosclerosis, coronary artery calcification, and carotid atherosclerosis [16]. The study of Mheid et al. [17] showed that VD deficiency was related to atherosclerosis and was related to endothelial



**Figure 3.** Serological markers and cardiac hematoxylin and eosin staining in rats. (A, C-F) Serum 25-OH-VD (A), TC (C), TG (D), HDL-C (E), and LDL-C (F) in different groups at different times. (B) Hematoxylin and eosin staining was used for coronary artery in the health (upper left), CHD (upper right), VD-CHD (lower left), and VD poor-CHD group (lower right) at 16 weeks.



**Figure 4.** Expression of cardiac inflammatory factors and SIRT1/p65 protein in rats. (A) The expression of inflammatory factors was measured by RT-qPCR; (B) The expression of SIRT1/p65 protein were measured by western blot. Compared with the CHD group, \*  $P < 0.05$  and \*\*\*  $P < 0.001$ ; compared with VD-CHD group, #  $P < 0.05$  and ###  $P < 0.001$ .

dysfunction, but the specific mechanism of VD deficiency on atherosclerosis was still unclear.

The current consensus is that atherosclerosis is a chronic inflammatory response of blood vessels. The inflammatory response runs through each of the processes of atherosclerosis plaque formation, progression, and rupture, and is an important driving factor in the development and progression of atherosclerotic lesions [1]. Therefore, we analyzed the relationship between serum 25-OH-VD and serum inflammatory factors in patients with CHD, and found that the level of serum 25-OH-VD in CHD patients had a negative correlation with

serum TNF- $\alpha$  ( $r = -0.651$ ,  $P < 0.001$ ), IL-6 ( $r = -0.457$ ,  $P < 0.001$ ), IL-8 ( $r = -0.755$ ,  $P < 0.001$ ) and IL-1 $\beta$  ( $r = -0.628$ ,  $P < 0.001$ ). On the one hand, studies have shown that VD deficiency can enhance vascular inflammatory responses due to the fact that VD can promote the secretion of anti-inflammatory cytokines, inhibit the secretion of inflammatory cytokines, and inhibit inflammation [18,19]. On the other hand, VD can also down-regulate the expression of endothelial cell adhesion molecules, and at the same time inhibit the activation of the endothelium induced by inflammatory reactions, thus exerting an anti-atherosclerotic effects [20,21].

These aforementioned studies present final results and we cannot know the dynamic relationship between serum 25-OH-VD and CHD, and cannot confirm the relationship between serum 25-OH-VD and inflammation. Therefore, we designed animal experiments to help further elucidate this issue. In animal experiments, we found that VD deficiency could increase the inflammatory response by upregulating the expression of p65 protein in the heart tissue and reducing SIRT1 protein expression, thereby inducing or aggravating the state of CHD, and VD supplementation could reverse this process.

NF- $\kappa$ B can regulate the transcriptional expression of cellular genes in both directions, and then participate in the occurrence and development of a series of diseases by controlling the expression of related genes such as autoimmune responses, inflammatory responses, and apoptosis. Studies have confirmed that NF- $\kappa$ B signaling pathway plays an important role in the occurrence and development of CHD [22,23], and high expression of NF- $\kappa$ B suggests that patients may have severe coronary artery stenosis [24] and plaque formation [25]. Studies have shown [26] that the plasma soluble vascular cell adhesion molecule level-1 (sVCAM-1) may contribute to the production of various atherosclerotic cytokines and chemokines by promoting the activation of NF- $\kappa$ B, and the formation and rupture of plaques ultimately contribute to the development of acute coronary syndromes. A large number of experiments have shown that the expression level of NF- $\kappa$ B related factors in the peripheral blood of the heart were closely related to the

inflammatory state and diseased progression of CHD and expected to become an important indicator for the detection of CHD [27]. In addition, animal experiments found that inhibit the conduction of NF- $\kappa$ B signaling pathway could not only reduce myocardial ischemia/reperfusion injury [28,29], but also effectively prevent the occurrence of CHD or achieve therapeutic effects [30,31]. Silence signaling factor 1 (SIRT1) is a widespread histone deacetylase that exists in human cells, and utilizes the activity of deacetylase to act on various transcription factors such as p53, UCP2, and NF- $\kappa$ B to play biological functions [32,33]. Since NF- $\kappa$ B protein p65 needs to be acetylated to exert its effect, SIRT1 uses its deacetylation to reduce acetylation of NF- $\kappa$ B p65 protein in inflammatory response, this in turn inhibits the transcriptional levels of inflammatory genes such as TNF- $\alpha$  and IL-6 downstream of NF- $\kappa$ B [34].

## Conclusions

Serum 25-OH-VD was reduced in CHD patients and it was a protective factor for CHD and negatively correlated with serum inflammatory factors. VD deficiency could increase the inflammation by upregulating the conduction of NF- $\kappa$ B signaling pathway in heart tissue of rats.

## Conflict of interest

None.

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