

Original Research Paper

Vitamin D Supplements Tone Down the Progression of Atheromatous Plaque Formation in a Dose-Dependent Manner

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Abstract: Hypovitaminosis D has a negative impact on the cardiovascular system. We performed the current study to investigate the expression of Vitamin D Receptor (VDR) in atherosclerotic human male aortas and to examine the effect of different doses of Vit. D supplementation on VDR expression in the aortas of male rats fed a High-Fat Diet (HFD). Human participants included 50 atherosclerotic male patients with anterior myocardial infarction and 131 control healthy males. Fifty male Wistar rats were divided into 2 groups: Control (Cont.) group; n = 10 and HFD fed rats; n = 40. The latter was divided equally into 4 subgroups according to the dose of Vit. D supplemented. Vitamin D was supplemented in 3 doses: (0.025, 0.05, 0.075 µg/kg) for a duration of 17 weeks. VDR expression score was immunohistochemically evaluated in aortas of both human and animal study groups. The current study revealed that Vit. D levels were significantly lower in atherosclerotic patients with myocardial infarctions compared to the human control group. Concurrent administration of Vit. D suppresses the progress of atheromatous plaque in rats fed a HFD in a dose-dependent manner. Consequently, it could be concluded that Vit. D supplements are recommended as a prophylaxis in patients at high risk of coronary artery disease.

Keywords: Vitamin D, Vitamin D Receptors, Atherosclerosis

Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity globally (Mozaffarian *et al.*, 2016). Hypovitaminosis D is proposed as a predisposing factor for many of them (Muscogiuri *et al.*, 2017). The active form of Vit. D acts through its Receptors (VDR) in cardiomyocytes to decrease the production of renin and the development of foam cells (Muscogiuri *et al.*, 2017). It inhibits cardiovascular muscle proliferation and dampens intracellular inflammatory processes.

Previous studies report contradictory results regarding the association between serum Vit. D levels and cardiovascular diseases (Muscogiuri *et al.*, 2017). There is much evidence that cardiovascular diseases (hypertension, atherosclerosis, myocardial infarction and heart failure) and diabetes mellitus are associated with

hypovitaminosis D (Nitsa *et al.*, 2018). Also, Vit. D deficiency has been found to promote atheromatous formation which could be reversed by Vit. D supplements (Carvalho and Sposito, 2015; Nsengiyumva *et al.*, 2015). Recently, vit. D deficiency (<15 ng/mL) has been recognized as a risk factor for several cardiovascular diseases, even in healthy people (Lee *et al.*, 2008; Rimondi *et al.*, 2021). On the other hand, some studies found that high levels of Vit. D increased the hazard of vascular calcifications (Abraham *et al.*, 2011; Razzaque, 2011).

To this end we performed the current study to compare serum Vit. D levels between atherosclerotic patients and healthy controls. We also explored VDR expression in aortas of atherosclerotic humans and rats fed a HFD. Finally, we evaluated the effect of supplementations of different Vit. D doses on VDR.

Patients, Animals and Methods

The current study was conducted in the Faculty of Medicine, Assiut University in collaboration with Al-Orman University Hospital between March 2018 and December 2019. The study included both human and animal participants. All procedures were implemented in agreement with the ethical principles of the Medical Ethics Committee, Faculty of Medicine, Assiut University IRB no. 17300511 and with the principles for Use and Care of Laboratory Animals accepted internationally. A written informed consent was taken and archived for all human study participants.

Human Participants

Our study included 50 male patients, aged between 23-66 years, suffering from extensive anterior infarction according to the investigations (cardiac markers, ECG, echocardiography and coronary angiography) and admitted to the Cardiothoracic Surgery Department for surgical interventions. All patients were heavy smokers and 17 of them were diabetic. Twenty were subjected to minimal invasive coronary artery bypass and 30 were subjected to open heart surgery for ordinary Coronary Artery Bypass Graft (CABG). These patients were compared to 131 age and sex matched controls.

All the human study's participants were subjected to routine investigations (random blood glucose level, glycosylated hemoglobin, lipogram, liver and renal function tests, coagulation profile and abdominal ultrasonography).

Three mL venous blood was collected from each human participant in a plain test tube left at room temperature for 10 min to be clotted and then centrifuged for 15 min at 3000 round per minute. The sera were separated and kept at -20°C till the time of analysis. Vit. D levels were measured by *EDI™* total 25-OH Vit. D ELISA kit (Epitope Diagnostic, Inc., San Diego, CA 92121, USA) according to the manufacturer protocol. Colorimetric methods were used to measure serum total cholesterol (Spectrum Diagnostic Egypt, Cat# 230003), HDL-C (Spectrum Diagnostic Egypt, Cat# 266002), serum triacylglycerol (Spectrum Diagnostic Egypt, Cat# 314003) and LDL-C by calculation. Two mg of tissues were removed from the aorta of atherosclerotic patients during the re-implantation of the new blood vessels and sent for histopathological studies and Vit. D receptor scoring.

Animals and Housing

Adult male Wistar rats weighing 100-150 g were purchased from the animal house (Faculty of Medicine, Assiut University). They were raised in a standard animal-grade room (22-24°C and 12 h light/dark cycles) with four to five rats in each cage with food (laboratory chow) and water *ad libitum*. They were kept in the animal house for one week before starting the experiments. Rats were divided into 2 groups; control (n = 10) fed on

standard laboratory diet and HFD (n = 40) fed a high fat diet (consisting of 40% energy from fat). The HFD group was subdivided into 4 subgroups (n = 10/group) based on Vit. D supplementation (ALPHA company, India, Batch No: 443099) dissolved in olive oil and administered via a gastric tube (Zhou *et al.*, 2018). The four subgroups are G1 (no Vit. D), G2 (low-dose Vit. D, 0.025 µg/kg) G3 (medium-dose Vit. D, 0.05 µg/kg) and G4 (high-dose vit D, 0.075 µg/kg).

Body weight was measured at the beginning of the experiment (initial body weight), immediately before starting Vit. D administration and on the day of euthanasia (final body weight). The Lee index was calculated as the cube root of body weight (g) divided by the naso-anal length (cm).

Two mL blood samples were collected from retroorbital veins from all groups in a plain test tube, centrifuged at 700 G for 10 min to remove blood cells. The sera were collected and stored at -20°C until assayed. Determination of serum total lipid was made by a commercial kit (Biodiagnostic, Egypt. Cat. No. 135:545). Vit. D levels were estimated using the same measuring method described for the human participants.

Histopathological preparation and staining of aortas were performed according to (Schnatz *et al.*, 2012) The animals were anesthetized with intraperitoneal thiopental (50 mg/kg) and sacrificed prior to the dissection of the abdominal aorta. Sections from the abdominal aorta were immediately fixed in 10% buffered formalin for 24 h, dehydrated in increasing concentrations of ethanol and processed for paraffin blocks. Sections (4 µm thick) were deparaffinized, stained with haematoxylin and eosin (H and E) and examined using an Olympus light microscope.

Immunohistochemical Staining of Human and Rodent Aorta Specimens

Using the avidin-biotin immunoperoxidase method, four µm thick sections were taken from previously prepared paraffin-embedded tissue blocks. The sections were deparaffinized and rehydrated with a graded ethanol series descending to distilled water. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide treatment. For epitope retrieval, sections were microwaved in citrate buffer (pH 6) for a total of 12 min. Sections were incubated with a primary antibody against VDR (Abcam, ab3508, diluted at 1/500) for 24 h at room temperature. Secondary staining kits were used according to the manufacturer's instructions (Thermo Scientific Corporation Fremont, CA, USA). Counterstaining was performed with H and E, then the sections were examined by light microscopy. The percentage of positive nuclear staining in the intima and media was evaluated. The intensity of staining was assigned to a grade of 0, 1, 2, or 3, where a grade 0 indicated a nucleus with no staining, a grade 1 indicated weak staining, a grade 2 indicated a moderate staining and a grade 3 indicated a strong staining. An immunohistochemical H-score was calculated by multiplying the percentage of positively stained nuclei and the grade of staining intensity.

Statistical Analysis

The data were analyzed by SPSS-v. 26 and expressed as mean \pm standard deviation. Means were compared statistically using One Way ANOVA test followed by Tukey for multiple comparisons. P-values <0.05 were considered statistically significant.

Results

Human Results

The current study found a significant increase in BMI, cholesterol, serum LDL-cholesterol, glucose and HbA1c levels and a significant decrease in serum Vit. D and HDL-cholesterol levels in atherosclerotic patients compared to controls (Table 1 and Fig. 1).

Histopathologic examination of sections from the abdominal aorta showed infiltration of the arterial wall by foamy cells, with well-formed atheromatous plaques were found (Fig. 2). Immunohistochemical staining with VDR

antibody revealed a significantly lower expression of VDR (Fig. 3).

Animal Results

There was insignificant difference between the HFD (127.52 \pm 18.65 gM) and the Cont. (124.05 \pm 17.76 gM) groups, regarding the initial weights. Immunohistochemical staining with VDR antibody revealed a significantly higher expression of VDR in the control group compared to the HFD, small-dose and medium-dose Vit. D groups (P = 0.0001). The large-dose Vit. D group showed significantly higher VDR expression compared to the HFD group (P = 0.0001) while the difference between its expression in the large-dose Vit. D group and control group was insignificant (P = 0.055) (Table 2).

Histopathologic examination of sections from the positive control showed infiltration of the arterial wall by foamy cells (Fig. 4). Immunohistochemical staining with VDR antibody revealed a significantly higher expression of VDR in the control group compared to the HFD, small-dose and medium-dose Vit. D groups (Fig. 5).

Table 1: Mean \pm SD of different studied variable in human participants.

	Control N = 131	Cases N = 50	p-value
Age in years	48.00 \pm 8.31	49.64 \pm 6.37	0.21
Body mass index (kg/m ²)	23.77 \pm 5.57	27.58 \pm 2.69	<0.001
Vitamin D level (ng/mL)	45.27 \pm 14.92	18.06 \pm 13.34	<0.001
Serum LDL-cholesterol (mg/dL)	56.08 \pm 6.67	188.30 \pm 83.917	<0.001
Serum HDL-cholesterol (mg/dL)	85.79 \pm 23.67	42.80 \pm 8.56	<0.001
Serum triacylglycerol (mg/dL)	133.11 \pm 30.274	136.72 \pm 107.41	0.73
Serum cholesterol (mg/dL)	164.79 \pm 32.98	261.22 \pm 87.764	<0.001
Glucose (mg/dL)	97.94 \pm 10.99	118.13 \pm 29.39	<0.001
HbA1c (%)	4.31 \pm 0.59	6.31 \pm 2.60	<0.001

Table 2: Comparison of different studied variables among the studied groups

		Control Group (G1)	HFD group (G2)	Small vitamin D group (G3)	Medium vitamin D group (G4)	Large vitamin D group (G5)
Score of vitamin D receptors expression	Mean \pm SD	255.0 \pm 13.4	70.0 \pm 80.2	72.5 \pm 21.4	88.0 \pm 39.6	198 \pm 32.5
	p-value		0.000	0.000	0.000	0.055
	Vs G1			0.115	0.516	0.000
	Vs G2				0.000	0.001
	Vs G3					0.000
LEE index (weight ^{0.33} /Naso-Anal Length) g/cm (at the end)	Mean \pm SD	0.31 \pm 0.01	0.34 \pm 0.01	0.35 \pm 0.01	0.34 \pm 0.02	0.36 \pm 0.02
	p-value		0.002	0.000	0.001	0.001
	Vs G1			0.374	0.496	0.103
	Vs G2				0.931	0.265
	Vs G3					0.496
Serum total lipid (mg/dL)	Mean \pm SD	248.50 \pm 38.88	448.86 \pm 20.09	430.83 \pm 14.29	398.00 \pm 10.37	325.00 \pm 11.18
	p-value		0.000	0.000	0.000	0.002
	Vs G1			0.094	0.000	0.000
	Vs G2				0.002	0.000
	Vs G3					0.000
Serum vitamin D level (ng/mL)	Mean \pm SD	82.00 \pm 16.44	52.57 \pm 11.84	73.83 \pm 12.66	86.00 \pm 14.11	85.80 \pm 13.66
	p-value		0.003	0.358	0.679	0.691
	Vs G1			0.010	0.001	0.001
	Vs G2				0.166	0.166
	Vs G3					0.001

SD: Standard deviation

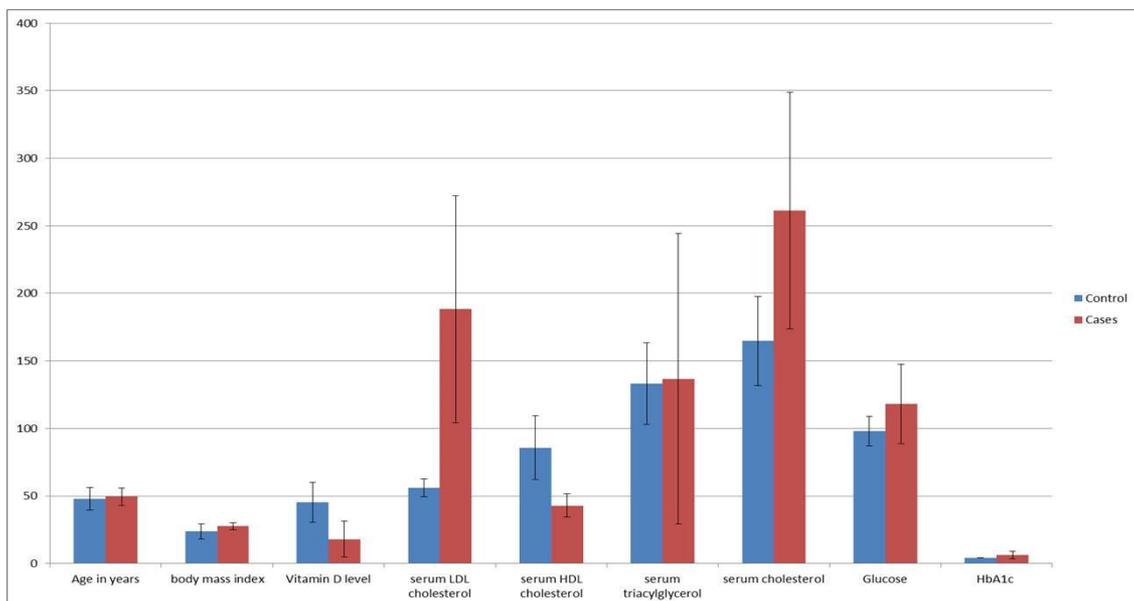


Fig. 1: Histogram showed mean \pm SD of different studied variable in human participants

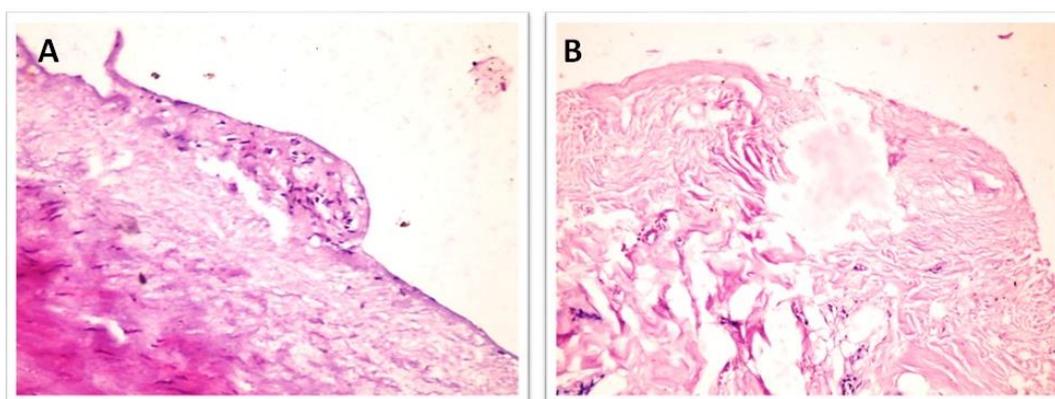


Fig. 2: Photomicrographs of specimens from human aorta stained with Haematoxylin and Eosin. A: Infiltration of subendothelial tissue by inflammatory cells. B: Large atheroma with cholesterol clefts and hyalinosis of subendothelial tissue.

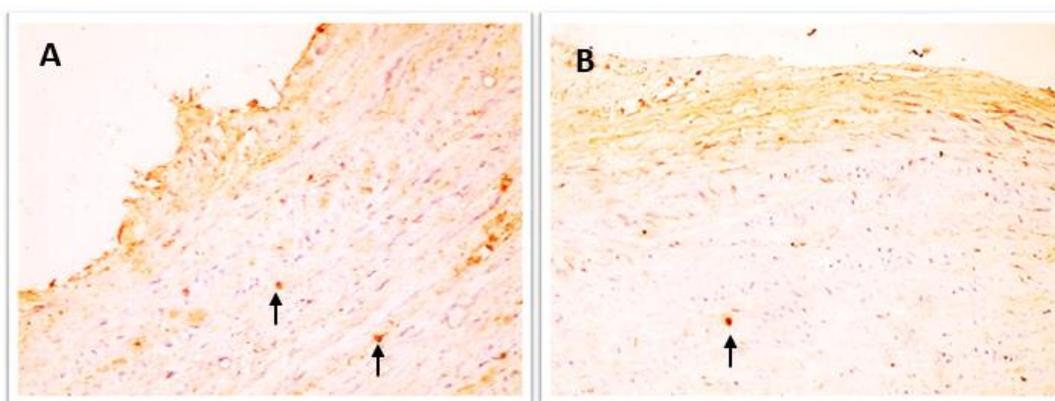


Fig. 3: A, B Photomicrographs of specimens from human aorta immunohistochemically stained for Vitamin D Receptors (VDR). Arrows point to the VDR stained nuclei

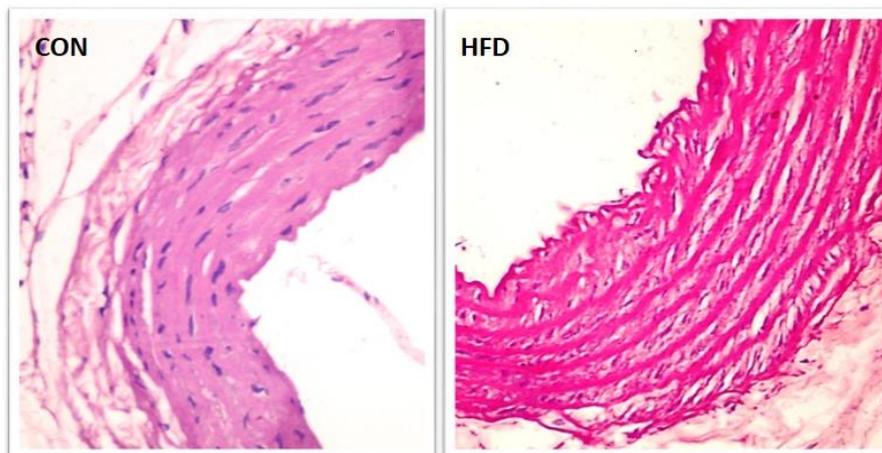


Fig. 4: Photomicrographs of the aorta of rats stained with Haematoxylin and Eosin. CONT: control group. HFD: High fat diet group showing early infiltration of the wall by foamy macrophages

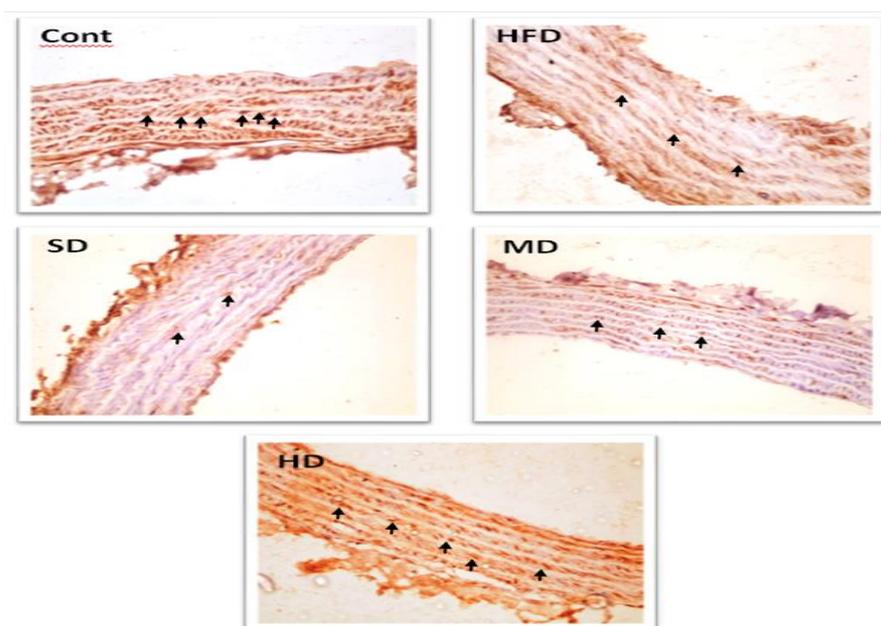


Fig. 5: Photomicrographs of the aorta stained immunohistochemically with VDR in different studied groups. Cont: Control group, HFD: High fat diet group, SD: Small dose vitamin D group, MD: Medium dose vitamin D group, HD: High dose vitamin D group. The arrows refer to the VDR stained nuclei

Discussion

Coronary artery disease is one of the most common cardiac disorders preceded by atherosclerosis. It is multifactorial and causes include genetic, environmental and life-style dysregulations. Deficiency of 25-hydroxy Vit. D has been identified as a predisposing factor for cardiovascular diseases in the population (Lee *et al.*, 2008; Rimondi *et al.*, 2021). This deficiency is associated with a state of systemic and vascular inflammation, vascular infiltration of foam cells and development plaque

(Gluba-Brzózka *et al.*, 2018; Junarta *et al.*, 2019). It also leads to accumulation of lipids and calcium (Nitsa *et al.*, 2018), proliferation in adventitial vasa vasorum, endothelial dysfunction and plaque instability (Rimondi *et al.*, 2021). Adjustment of Vit. D levels slowdown the progression of these cardiovascular consequences (Rimondi *et al.*, 2021; Junarta *et al.*, 2019). Vit. D suppresses the production of TNF- α , interleukin-6, 1, 8 and decreases the acute phase inflammatory reactions which slowdown the progress of atherosclerosis and plaque formation (Chen *et al.*, 2016). Also, Vit. D reduces cholesterol accumulation and low

density protein uptake by endothelial cells (Yin *et al.*, 2015). Likewise, Vit. D regulates the vascular tone by upregulating endothelial Nitric Oxide Synthase (eNOS), downregulation of endothelial Cyclooxygenase1 (COX-1) and reduction of reactive oxygen species (Ni *et al.*, 2014). Vit. D receptors have been reported to have pivotal role in the regulation of endothelial functions through their effect on rennin-angiotensin system. The VDR knockout rats were found to have a significant high incidence of developing hypertension and cardiac hypertrophy when compared to the control group due to induction of rennin expression and activation of renin-angiotensin pathway (Latic and Erben, 2020).

The current study revealed significant decrease in Vit. D levels in atherosclerotic patients and in animals fed on HFD. Similarly, Chen *et al.* 2016 reported that Vit. D deficiency enhances coronary atherosclerosis by activation of KB (NFKB) which accelerates the progression of atherosclerosis and also confirmed the anti-inflammatory functions of Vit. D (Chen *et al.*, 2016). Moreover, the present study reported a significant decrease in serum total lipids in rats after Vit. D supplementation which is in agreement with Sharma *et al.* (2010) who described a reduction in cholesterol and LDL levels in macrophages after Vit. D supplementations (Sharma *et al.*, 2010).

Furthermore, In the present study, the aortic atheromatous formation was associated with Vit. D deficiency which comes in agreement with Rimondi *et al.*, (2021) who reported a promotion of atheromatous plaque formation and instability in participants with Vit. D deficiency. The plaque had plenteous lipid center with a tinny fibrous cap. This fibrous cap was found to contain small number of smooth muscle cells when compared to the control group (Luo *et al.*, 2017).

In the present work, Vit. D supplements increase Vit. D receptors score in animals fed on HFD. Our findings agree with those of Farrokhian *et al.* (2017) who reported that Vit. D supplement was beneficial against vascular inflammation and also increased nitric oxide in diabetic patients. Moreover, Sokol *et al.* (2012) reported that Vit. D daily supplement of 4000 IU for 5 days in myocardial infarction patients attenuated CRP and interleukin-6 (Sokol *et al.*, 2012). However, Scragg *et al.* (2017) claimed that monthly supplement of 10000 IU Vit. D over 3 years didn't have any beneficial impact on coronary artery diseases (Scragg *et al.*, 2017).

The current study shows an early infiltration of the rats' aortic wall by foamy macrophages associated with a decrease in Vit. D receptors. However, the administration of different doses of Vit. D to those rats increases the Vit. D receptor score significantly and this increment was positively correlated with the supplemented dose of Vit. D in agreement with previous studies (Rimondi *et al.*,

2021; Carlberg, 2016; Christakos *et al.*, 2016). Thus, the use of Vit. D was proposed to prevent many cardiovascular disorders including atheroma formation. Recently, Munshi *et al.* (2021) concluded that the use of Vit. D supplements is valuable in the prevention of cardiovascular pathologies not only in chronically ill patients but also in the general population (Munshi *et al.*, 2021). This, in turn, leads to a significant decrease in the health insurance costs and improvement in the life quality of patients.

Conclusion

Vit. D levels were significantly low in atherosclerotic patients with myocardial infarctions. Also, concurrent administration of Vit. D suppresses the progress of atheromatous plaque in rats feed on high fat diet in a dose dependent manner.

Recommendation

Vit. D supplement is recommended in all cases presented with one or more predisposing factors for atherosclerosis and other coronary artery diseases

Limitation of Study

The duration of Vit. D supplementation (17 weeks) was not enough to get the optimal data regarding the atherosclerotic changes and the VDR.

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Author's Contributions

Ahmed Farouk: Sharing in Idea, Doing Surgery, Help in writing.

Khalid M. Mohany: Statistical, Writing, Editing.

Magdy Algowhary: Idea, Catheter, Clinical Ex.

Lobna A. Abdelzaher: Care of animal dose, Adjustment.

Heba E. M. El-Deek and Ghada Hosny: Histopathology work, Writing.

Tahia Hashim Saleem: Idea, Biochemistry work, Writing, Editing.

Ethics

All procedures were implemented in agreement with the ethical principles of the Medical Ethics Committee, Faculty of Medicine, Assiut University IRB no. 17300511 and with the principles for Use and Care of Laboratory Animals accepted internationally. A written informed consent was taken and archived for all human study participants.

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