

Vitamin D Replacement Mitigates Menopause-Associated Dyslipidaemia and Atherogenic Indices in Ovariectomized Rats; A Biochemical Study

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Key words

Ovariectomized rats, vitamin D status, dyslipidaemia, atherogenic indices, ABCA1/JNK/CD36

received 04.11.2018

revised 26.03.2019

accepted 22.05.2019

Bibliography

DOI <https://doi.org/10.1055/a-0934-5666>

Published online: 2019


Exp Clin Endocrinol Diabetes 2019; 127: 1–8

© J. A. Barth Verlag in Georg Thieme Verlag KG Stuttgart · New York

ISSN 0947-7349

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 **Supplementary Material** for this article is available online at <http://www.thieme-connect.de/products>.

ABSTRACT

Background & Aim Dyslipidaemia is highly prevalent among postmenopausal women and its management represents a keystone in the prevention of the worldwide increase in cardiovascular morbidity and mortality. Therapy choices for menopause-associated dyslipidaemia are limited and a matter of debate. So, it becomes prudent to search for natural safe alternatives. Vitamin D (VD) has been acknowledged as an essential factor in cardiovascular health. Thus, we aimed to illustrate the impact of different VD status on dyslipidaemia and atherogenic indices.

Method 5 groups of rats were conducted; SHAM group fed control diet, ovariectomized rats fed control diet (OVX), ovariectomized rats fed VD-sufficient-high fat diet (HFD) (1 000 IU/Kg diet), ovariectomized rats fed VD-deficient-HFD (25 IU/kg diet), and ovariectomized rats fed VD-replete-HFD (10 000 IU/kg diet) for 16 weeks.

Results Dyslipidaemia with an increased atherogenic index of plasma, atherosclerosis coefficient, cardiac risk ratio, and aortic total cholesterol accumulation in addition to reduced serum 25-hydroxy-VD levels was observed in the OVX and VD-sufficient HFD versus SHAM. These findings were aggravated by VD-deficient-HFD while reversed by VD-replete-HFD. The VD-mediated abundance of aortic ATP-binding cassette transporter A1 (ABCA1) expression, reduced activity of the inflammatory Jun N-terminal kinases (JNK), and downregulation of aortic cluster of differentiation-36 (CD36) receptors expression together with increased serum total antioxidant capacity and reduced serum malondialdehyde were among the supposed mechanisms.

Conclusions Our study sheds light on alarming levels of VD deficiency among ovariectomized rats. VD repletion improved the menopause-associated dyslipidaemia and atherogenic indices through hypolipidemic, antioxidant, and anti-inflammatory effects.

ABBREVIATIONS

| | |
|--------|--|
| ABCA1 | ATP binding cassette transporter A1 |
| AC | atherogenic coefficient |
| AIP | atherogenic index of plasma |
| CD36 | cluster of differentiation 36 receptor |
| CRR | cardiac risk ratio |
| CVD | cardiovascular disease |
| HDL-C | high density lipoprotein-cholesterol |
| HFD | high fat diet |
| JNK | Jun N- terminal kinases |
| LDL-C | low density lipoprotein-cholesterol |
| LDL-R | low density lipoprotein-receptors |
| MDA | malondialdehyde |
| OVX | ovariectomy |
| TC | total cholesterol |
| TAC | total antioxidant capacity |
| TGs | triglycerides |
| VD | vitamin D |
| VDD | vitamin D deficiency |
| VLDL-C | very low density lipoprotein-cholesterol |

Introduction

Menopause is a form of reproductive ageing that represents a normal phase of a woman's life cycle [1]. Atherosclerotic cardiovascular diseases (CVD) are critically increased in the postmenopausal women and identified as the foremost cause of morbidity and mortality [2]. Dyslipidaemia with elevated serum cholesterol is a key player in the pathogenesis of atherosclerosis [3]. Thus, understanding of cholesterol trafficking is essential to combat it.

Low-density lipoprotein-cholesterol (LDL-C) "bad cholesterol" transports cholesterol to peripheral tissues where it enters the cells through LDL receptor (LDL-R) [4]. When oxidized by free radicals, oxidized LDL-C particles internalisation is shifted to another receptors named "cluster of differentiation 36" (CD36) [5, 6]. Unlike to LDL-R, CD36 are not downregulated by the excess intracellular cholesterol leading to massive cholesterol accumulation and lipotoxicity [7]. This subsequently, triggers an inflammatory reaction with Jun N-terminal kinases (JNK), are involved. They are signaling molecules members of the mitogen-activated protein kinase superfamily. They are triggered by stressors such as redox imbalance, fatty acids, and inflammatory cytokines and predispose to atherosclerotic events [8, 9].

Also, high-density lipoprotein-cholesterol (HDL-C) "good cholesterol" plays a role in cholesterol homeostasis; it removes excess free cholesterol and oxidized lipids from peripheral tissues back to the liver to be bile excreted [10]. Thus, it is considered a vital cardiovascular protective agent under hypercholesterolemic condition [11]. ATP binding cassette transporter A1 (ABCA1) is a lipid transporter protein that mediates the cholesterol efflux to HDL-C. So, it is a key player in cardiovascular and metabolic disease at local and systemic level [12]. Accordingly, HDL-C increase and LDL-C decrease provide the framework for managing dyslipidaemia [13]. Moreover, lipid profile ratios that associate the atherosclerotic process are known as atherogenic indices. They include the athero-

genic index of plasma (AIP), atherosclerosis coefficient (AC), and cardiac risk ratio (CRR) and can predict the CVD risks [14].

Existing guidelines for dyslipidaemia management in postmenopausal women recommend statins and hormonal replacement therapy (HRT). Although statins showed progress in reducing the risk, many patients still have residual cardiovascular hazards with an increased incidence of diabetogenesis in women [15]. Furthermore, the American Heart Association recently, recommends against the use of HRT to minimize the hazard of coronary heart disease and stroke [16]. So, it becomes prudent to search for natural safe alternatives or supplements.

Vitamin D (VD) has been acknowledged as an essential factor in cardiovascular health beyond its well-defined role in calcium homeostasis. It's essential for maintenance of endothelial health, modulation of immune function, and arrest vascular smooth muscle growth [17]. Notably, vitamin D deficiency (VDD) has arisen as a worldwide health concern. It is widely prevalent in elderly females about 50–80% [18]. Thus, VDD has been proposed to be related to a number of CVD.

Accordingly, we aimed to illustrate the effect of different dietary VD interventions on serum lipid profile alterations and atherogenic indices in an experimental rat model of menopause, with possible involvement of aortic CD36 receptors and ABCA1 transporter expressions with JNK activity and redox status as suggested mechanisms.

Materials and Method

Ethics statement

The experimental procedures, animal handling, sampling, and scarification were done according to the Guide for the Care and Use of Laboratory Animals, Eighth Edition (2011) and all efforts were made to minimize the animals suffering. The protocol was revised and approved by the Ethical Committee of Animal Experiments, Faculty of Medicine, Benha University, Egypt.

Experimental design and groups

Thirty female albino Wistar rats, 13-weeks-old, weighing 180 ± 10 g were purchased from Animal House, Faculty of Veterinary Medicine, Benha University, Egypt. They were housed in metallic cages and were maintained on prevailing atmospheric conditions and room temperature with free access to food and water for 2 weeks, as an acclimatization period. Thereafter, the rats were subjected to ovariectomy (OVX) or SHAM surgery. Both procedures were done under general anaesthesia using an intraperitoneal injection of pentobarbital sodium (40 mg/kg). The ovaries were bilaterally clamped and removed in OVX groups while only manipulated and not removed in the SHAM group. After surgery, the rats were maintained under cover of antibiotics and analgesics and good conditions for another 2 weeks [19].

The experimental diets were formulated and prepared as pellets at the Faculty of Agriculture, Benha University, Egypt, according to the following ingredients; Control diet, 16.99 MJ/kg metabolizable energy with 10% of its energy from fat, 70% from carbohydrates, 20% from protein, and recommended VD content, (1 000 IU/kg diet) [20]. High fat diet (HFD) was supplied to accelerate and augment lipogenesis process in ovariectomized rats [21]. HFD, 16.99 MJ/kg metabolizable energy, 45% of its energy is fat derived

mainly as lard with cholesterol content 127.8 mg/4057 kcal, 35% from carbohydrates in which corn starch was substituted by sucrose, 20% protein, and 1 000 IU VD /kg diet for VD-sufficient HFD, 25 IU VD /Kg diet for VD-deficient HFD, and 10 000 IU VD /Kg diet for VD-replete HFD [22] (**Supplemental Table 1S**). The dietary VD contents were as recommended by The National Research Council [20] that stated requirement for rodent serum VD level to be optimal is 1000 IU VD/kg diet, regardless of fat content. By contrast, the 25 IU VD/kg diet led to VD insufficiency.

The rats were then, divided according to the dietary manipulations into 5 groups (n = 6) as follow:

Group I, SHAM group, consisted of sham-operated rats fed control diet for 16 weeks; Group II, OVX group, consisted of ovariectomized rats fed control diet for 16 weeks; Group III, VD-sufficient group, consisted of ovariectomized rats fed VD-sufficient HFD for 16 weeks; Group IV, VD-deficient group, consisted of ovariectomized rats fed VD-deficient HFD for 16 weeks; Group V, VD-replete group, consisted of ovariectomized rats fed VD-replete HFD for 16 weeks [22]. Animals had free access to food and water and were kept at room temperature with restriction of sunlight and 12h fluorescent light/dark cycle.

The body weight and waist circumference were weekly assessed. At the end of 16-weeks and after an overnight fasting, the rats were sacrificed under general anaesthesia using an intraperitoneal injection of pentobarbital sodium (40 mg/ kg). The blood was collected by cardiac puncture. The uterus and heart were removed, washed and weighed. Aortic tissue was dissected carefully from the surrounding connective tissue and placed into ice-cold phosphate buffered saline then stored at -80°C for, JNK activity, Real-Time PCR analysis and aortic total cholesterol (TC) measurements.

Serum preparation

Collected blood samples were protected from direct sunlight and left to be clotted then centrifuged at 3000 rpm for 15 min. Serum was collected and divided into 2 parts; one for VD assessment that and the other part for lipid profile and redox status parameters. Both samples were stored at -20°C till analysis.

Serum 25-OH VD and redox status parameters assay

By following the manufacturer's instructions, rat 25-OH VD ELISA kits, (MyBioSource, CA, USA), MBS728692, were used to estimate serum 25-OH VD concentration.

Rats' malondialdehyde (MDA) and total antioxidant capacity (TAC) colorimetric assay kits purchased from (Abcam, Cambridge, UK), ab118970 and ab65329 respectively, were used to assess the rats' oxidant status according to the manufacturer's instruction.

Serum lipids profile assessment and atherogenic indices calculation

Triglycerides (TGs), total cholesterol (TC), and HDL-C enzymatic colorimetric assay kits (Diamond Diagnostic, Cairo, Egypt) were used. Very low-density lipoprotein-cholesterol (VLDL-C) was calculated as follow: $\text{VLDL-C} = \text{TGs}/5$ [23] while, LDL-C was ascertained according to the Friedewald et al. equation: $\text{LDL-C (mg/ dL)} = \text{TC} - \text{HDL-C} - (\text{TGs}/5)$ [24].

The atherogenic indices were calculated using the following established formulas: $\text{AIP} = \text{Log (TGs/HDL-C)}$, $\text{AC} = (\text{TC} - \text{HDL-C})/\text{HDL-C}$, and $\text{CRR} = \text{TC}/\text{HDL-C}$ [25].

Aortic TC content and JNK-activity assay

The accumulation of cholesterol in the aorta was measured using total cholesterol kits, (Diamond Diagnostic, Cairo, Egypt). Concisely, part of frozen aortic tissue was freeze-dried and weighed. The lipids were then extracted at 50°C for 20 min with chloroform-methanol (2:1). The total cholesterol was measured in that extract. The results were expressed as mg/g dry tissue [26].

To estimate JNK activity in the aortic tissue, rat c-JNK kinase ELISA kits, (MyBioSource, CA, USA), MBS2605744, were used by following the manufacturer's instructions.

Real-Time PCR analysis

Aortic tissue CD36-receptors and ABCA1-transporter were analysed. In brief, part of the aortic tissue was cut then homogenised using a Mixer Mill MM400 (Retsch, Germany). Total RNA was prepared using Trizol reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's protocol. The cDNA was synthesized by the two-step RT-PCR method (Prime Script RT Reagent Kit; Perfect Real Time; TaKaRa Code RR037A, Otsu, Japan). Temperature cycles were as follows: 95°C for 30 s followed by 42 cycles of 95°C for 5 s and 60°C for 30 s. The SYBR Green fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. The relative quantities of the CD36 and ABCA1 genes were normalized against the relative quantities of the endogenous control β -actin housekeeping gene. Fold expression changes are calculated using the equation $2^{-\Delta\Delta\text{ct}}$ [27]. Rats' oligonucleotide sequences of both forward and reverse primers (Operon, Inc., Huntsville, Alabama, Germany) were as follow:

CD36 F: AGGAAGTGGCAAAGAATAGCAG and R: ACAGACAGTGAAGGCTCAAAGA,
ABCA1 F: CCCGGCGGAGTAGAAAGG and R: AGGGCGATGCAAACAAAGAC,
 β -actin F: CACCCGCGAGTACAACCTT and R: CCCATACCCACCATCACACC.

Statistical analysis

All data were analysed using the program Statistical Package for Social Sciences (SPSS) version 19 (SPSS Inc., Chicago, IL, USA). They are presented as the mean \pm standard deviation. Comparisons of all parameters among the study groups were analysed by using one-way analysis of variance (ANOVA) test and post hoc multiple comparisons (LSD test). Multivariate linear regression analysis was performed to detect the association between serum 25-OH VD levels and atherogenic indices. The probability of chance (P value) < 0.05 was considered statistically significant.

Results

Effect of ovariectomy surgery and VD dietary intervention on anthropometric parameters in experimental groups (► Table 1)

A significant reduction in uterine weights was documented in all ovariectomized rat in the OVX, VD-sufficient, VD-deficient, and VD-replete groups when compared to the SHAM group ($P < 0.05$). Moreover, significant increases in the body weight, waist circumference, and heart weight were observed in the OVX and VD-suffi-

► **Table 1** Anthropometric parameters and serum 25-OH VD levels in experimental groups.

| Groups | SHAM | OVX | VD-sufficient | VD-deficient | VD-replete |
|--------------------------|--------------|----------------------------|-----------------------------|------------------------------|---------------------------------|
| Body weight (g) | 209 ± 7 | 300 ± 6 ^{§~} | 408 ± 17 ^{#§} | 517 ± 34 ^{# * §~} | 249 ± 10 ^{# * §~} |
| Waist circumference (cm) | 10.76 ± 0.6 | 12.6 ± 0.9 ^{§~} | 15.13 ± 1.2 ^{#§} | 19.9 ± 1 ^{# * §~} | 11.8 ± 0.67 ^{* §~} |
| Uterine weight (mg) | 501 ± 7 | 102 ± 2 [§] | 105 ± 4 [§] | 102 ± 5 [§] | 101 ± 4 [§] |
| Heart weight (mg) | 818 ± 11 | 889 ± 18 ^{§~} | 981 ± 23 ^{#§} | 1107 ± 31 ^{# * §~} | 850 ± 20 ^{# * §~} |
| 25-OH VD (nmol/L) | 63.47 ± 10.1 | 24.76 ± 3.93 ^{§~} | 15.67 ± 3.04 ^{#§~} | 9.85 ± 1.82 ^{# * §} | 107.76 ± 6.68 ^{# * §~} |

Data are expressed as mean ± standard deviation; n = 6; P value = probability of chance, [§]P < 0.05 significant difference compared with the SHAM group, [#]P < 0.05 significant difference compared with the OVX group, ^{*}P < 0.05 significant difference compared with the VD-sufficient group while, [~]P < 0.05 significant difference compared with the VD-deficient group. SHAM, sham-operated group; OVX, ovariectomy; VD, vitamin D; 25-OH VD, 25-hydroxy vitamin D

► **Table 2** Serum lipid profile parameters and calculated atherogenic indices in different experimental groups.

| Groups | SHAM | OVX | VD-sufficient | VD-deficient | VD-replete |
|----------------|--------------|----------------------------|----------------------------|---------------------------------|--------------------------------|
| TGs (mg/dl) | 31.88 ± 4.96 | 55.73 ± 4.31 ^{§~} | 79.33 ± 7.71 ^{#§} | 106.6 ± 3.37 ^{# * §~} | 40.97 ± 3.36 ^{# * §~} |
| TC (mg/dl) | 48.83 ± 2.99 | 75.17 ± 8.23 ^{§~} | 97.5 ± 4.51 ^{#§} | 127.33 ± 5.05 ^{# * §~} | 73.3 ± 2.16 ^{# * ~} |
| VLDL-C (mg/dl) | 6.38 ± 0.99 | 11.15 ± 0.86 ^{§~} | 15.87 ± 1.54 ^{#§} | 21.23 ± 0.69 ^{# * §~} | 8.19 ± 0.67 ^{# * §~} |
| HDL-C (mg/dl) | 29.83 ± 3.43 | 24.00 ± 3.74 ^{§~} | 20.00 ± 2.1 ^{#§} | 15.00 ± 2.83 ^{# * §~} | 40.67 ± 3.08 ^{# * §~} |
| LDL-C (mg/dl) | 12.62 ± 1.62 | 40.00 ± 7.47 ^{§~} | 61.63 ± 6.95 ^{#§} | 91.1 ± 6.93 ^{# * §~} | 24.48 ± 1.66 ^{# * §~} |
| AIP | 0.027 ± 0.11 | 0.369 ± 0.07 ^{§~} | 0.599 ± 0.06 ^{#§} | 0.859 ± 0.08 ^{# * §~} | 0.003 ± 0.04 ^{# * ~} |
| AC | 0.65 ± 0.11 | 2.18 ± 0.53 ^{§~} | 3.94 ± 0.7 ^{#§} | 7.83 ± 2.18 ^{# * §~} | 0.81 ± 0.1 ^{# * ~} |
| CRR | 1.65 ± 0.11 | 3.18 ± 0.53 ^{§~} | 4.94 ± 0.7 ^{#§} | 8.83 ± 2.18 ^{# * §~} | 1.81 ± 0.1 ^{# * ~} |

Data are expressed as mean ± standard deviation; n = 6; P value = probability of chance, [§]P < 0.05 significant difference compared with the SHAM group, [#]P < 0.05 significant difference compared with the OVX group, ^{*}P < 0.05 significant difference compared with the VD-sufficient group while, [~]P < 0.05 significant difference compared with the VD-deficient group. SHAM, sham-operated group; OVX, ovariectomy; VD, vitamin D; TGs, triglycerides; TC, total cholesterol; VLDL-C, very low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; AIP, atherogenic index of plasma; AC, atherogenic coefficient; CRR, cardiac risk ratio.

cient groups when compared to SHAM group. Additionally, these parameters were further aggravated in the VD-deficient group when compared to VD-sufficient group ($P < 0.05$). On the contrary, the VD-replete group exhibited significant reduction in body weight, waist circumference, and heart weight when compared to the OVX, VD-sufficient and VD-deficient groups ($P < 0.05$).

Effect of surgically-induced menopause and VD dietary intervention on serum 25-OH VD levels (► Table 1)

We observed that serum 25-OH VD level was significantly decreased in the OVX and VD-sufficient groups with respect to the SHAM group. Moreover, serum 25-OH VD levels were significantly the lowest in the VD-deficient group and highest in the VD-Replete group when compared to other groups ($P < 0.05$). Noteworthy, the serum levels of 25-OH VD in the VD-sufficient group was significantly lower than that of OVX group ($P < 0.05$).

Effect of surgically-induced menopause and VD dietary intervention on serum lipid profile in experimental groups (► Table 2)

Significant increases in the LDL-C, VLDL-C, TC, and TGs levels were noted in the OVX and VD-sufficient groups when compared to the SHAM ($P < 0.05$). The VD-deficient group exhibited further significant increases with respect to the OVX or VD-sufficient group ($P < 0.05$). Conversely, the VD-replete group showed significant decreases in LDL-C, VLDL-C, and TGs levels when compared to other

groups. TC was significantly decreased with respect to the OVX, VD-sufficient or VD-deficient groups ($P < 0.05$).

Regarding HDL-C levels, a significant decrease was observed in the OVX and VD-sufficient groups versus SHAM. Also, the VD-deficient group showed additional decreases in the HDL-C levels when compared to the OVX or VD-sufficient groups ($P < 0.05$). On the other hand, the VD-replete group exhibited significant increases in the HDL-C levels compared to the OVX, VD-sufficient or VD-deficient groups ($P < 0.05$).

Effect of surgically-induced menopause and VD dietary intervention on calculated atherogenic indices and their association to serum 25-OH VD levels (► Table 2, 3)

The AIP, AC, and CRR atherogenic indices were significantly increased in the OVX and VD-sufficient groups with respect to the SHAM ($P < 0.05$). They were further aggravated in the VD-deficient group versus the OVX or VD-sufficient group. On the other side, VD-replete group showed significant decline in these atherogenic indices when compared to OVX, VD-sufficient or VD-deficient groups ($P < 0.05$).

The multivariate linear regression analysis of the association between serum 25-OH VD levels and the atherogenic indices, revealed an inverse significant correlation between 25-OH VD levels and AIP ($P < 0.000$) and positive association with AC and CRR ($P < 0.000$) with the AIP is the most predictor for serum VD levels.

Effect of surgically-induced menopause and VD dietary intervention on aortic TC content (► Table 4)

The aortic TC content was significantly increased in the OVX and VD-sufficient groups versus the SHAM ($P < 0.05$) and it was further exaggerated in the VD-deficient group when compared to the OVX or VD-sufficient group ($P < 0.05$). Inversely, the VD-replete group exhibited a significant decrease in the aortic TC content versus the OVX, HFD or VD-deficient groups ($P < 0.05$).

Effect of surgically-induced menopause and VD dietary intervention on serum MDA and TAC and aortic JNK activity (► Table 4)

A significant increase in serum MDA with a significant decrease in TAC was exhibited in the OVX and VD-sufficient groups versus the SHAM group ($P < 0.05$). These observed changes were further aggravated in the VD-deficient group when compared to the OVX or VD-sufficient groups ($P < 0.05$). Contrarily, the VD-replete group exhibited significant MDA decrease and TAC increase when compared to the OVX, VD-sufficient or VD-deficient groups ($P < 0.05$).

The JNK activity was significantly increased in the OVX and VD-sufficient groups when compared to the SHAM group ($P < 0.05$). Also, the VD-deficient group showed a further significant increase in the JNK activity when compared to the OVX or VD-sufficient groups ($P < 0.05$). On the other hand, the VD-replete group resulted in a significant reduction in the JNK activity when compared to the OVX, VD-sufficient or VD-deficient groups ($P < 0.05$).

Effect of surgically-induced menopause and VD dietary intervention on aortic CD36 and ABCA1 mRNA expressions (► Fig. 1)

A significant up-regulation in the aorta CD36 mRNA and down-regulation in ABCA1 mRNA expressions were observed in the OVX

► **Table 3** Multivariate linear regression analysis between serum 25-OH VD and atherogenic indices in experimental groups.

| Variables | | Un-Standardized β -coefficients | Sig. |
|---|-------------|---------------------------------------|-------|
| Dependent | Independent | | |
| 25-OH VD | AIP | -674.7 | 0.000 |
| | CRR | 21.35 | 0.000 |
| | AC | 21.35 | 0.000 |
| (P value) | | (0.000) | |
| R ² (adj. r ²) | | 0.78 (0.75) | |
| 25-OH VD, 25-hydroxy vitamin D; AIP, atherogenic index of plasma; AC, atherogenic coefficient; CRR, cardiac risk ratio. | | | |

► **Table 4** Serum MDA and TAC and Aortic TC content and JNK activity in experimental groups.

| Groups | SHAM | OVX | VD-sufficient | VD-deficient | VD-replete |
|---|--------------|-----------------------------|------------------------------|-----------------------------|----------------------------|
| Aorta TC (mg/g dry tissue) | 6.7 ± 0.54 | 8.67 ± 0.43 [§] ~ | 11.53 ± 0.79 ^{#§} ~ | 15.57 ± 0.88 ^{#*§} | 6.93 ± 0.54 ^{#*~} |
| MDA (nmol/mL) | 1.78 ± 0.36 | 2.9 ± 0.28 [§] ~ | 3.9 ± 0.31 ^{#§} ~ | 5.16 ± 0.45 ^{#*§} | 2.05 ± 0.18 ^{#*~} |
| TAC (nmol/mL) | 8.78 ± 0.53 | 7 ± 0.47 [§] ~ | 5.8 ± 0.7 ^{#§} ~ | 2.5 ± 0.63 ^{#*§} | 8.2 ± 0.95 ^{#*~} |
| JNK (U/ml) | 30.57 ± 3.38 | 42.17 ± 4.58 [§] ~ | 51.75 ± 3.74 ^{#§} ~ | 65.73 ± 4.67 ^{#*§} | 33.4 ± 3.27 ^{#*~} |
| Data are expressed as mean ± standard deviation; n = 6; P value = probability of chance, [§] P < 0.05 significant difference compared with the SHAM group, [#] P < 0.05 significant difference compared with the OVX group, [*] P < 0.05 significant difference compared with the VD-sufficient group while, [~] P < 0.05 significant difference compared with the VD-deficient group. SHAM, sham-operated group; OVX, ovariectomy; VD, vitamin D; TC, total cholesterol; MDA, malondialdehyde; TAC, total antioxidant capacity; JNK, c-Jun N-terminal Kinase | | | | | |

group versus the SHAM group ($P < 0.05$). Their pattern of expression was further aggravated with the HFD and VD-deficient dietary groups when compared to the OVX group ($P < 0.05$) while it was reversed in the VD-replete dietary group ($P < 0.05$).

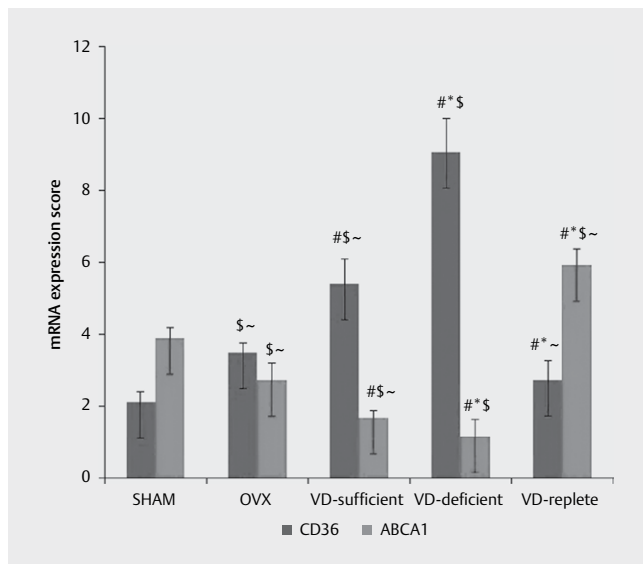
Discussion

The data of the present study revealed that sudden cessation of ovarian hormones caused by OVX surgery resulted in significant declines in the uterine weights of all ovariectomized rat groups when compared to SHAM group, indicting the effectiveness of OVX surgery. Later, we noticed that rats of OVX and VD-sufficient groups exhibited significant increases in the body weight and waist circumference when compared to SHAM group, denoting obesity with intra-abdominal fat accumulation. The increased adiposity was owing to menopause-associated declines in estrogen level, physical activity, and muscle fat oxidation [28]. With further fat re-distribution from subcutaneous to intra-abdominal visceral depots resulted in increased waist circumference that linked to significant CVD risks [29]. Those findings were in agreement with Ludgero-Correia et al. [19].

Our data also, revealed lower serum 25-OH VD levels in the OVX and VD-sufficient groups when compared to SHAM group, clarifying that menopause transition and HFD ingestion is a risk for VDD. Estrogen decline [30] and the associated ageing process [31] were suggested as causes to diminish VD-synthesizing enzymes activity. Furthermore, VD is sequestered in the enlarged adipose tissue of ovariectomized rats, being fat soluble, causing reduction of its serum bioavailability [32]. Also, seasonal variations of sunlight ultraviolet exposure and increasing indoor activities reduced cutaneous synthesis of VD [33]. In line with this finding, Babaei et al. reported VD insufficiency in ovariectomized rats [34].

Regarding serum lipid profile, our results showed dyslipidaemia that comprised of significant TGs, TC, LDL-C and VLDL-C increases with HDL-C decreases in the OVX and VD-sufficient groups when compared to the SHAM. This could be explained by that estrogen decline led to an alteration in peroxisome proliferator-activated receptor- δ expression important for fat metabolism [35], impaired synthesis of apolipoproteins essential for HDL formation, and lipoprotein lipases that utilize TGs causing impaired lipid profile [36].

Additionally, the atherogenic indices AC, CRR, and AIP that are group of lipid profile ratios linked to atherogenesis [25] were also affected. AC, being calculated as the ratio of non-HDL-C to HDL-C, [(TC - HDL-C)/HDL-C], reveals the atherogenic potential of lipoprotein fractions. Moreover, CRR, [TC/HDL-C], has been associated



► **Fig. 1** Effect of surgically-induced menopause and VD dietary intervention on aortic CD36 and ABCA1 mRNA expressions in experimental groups. Data are expressed as mean \pm standard deviation SD; $n=6$; mRNA expression data are Log_{10} relative units of relative quantitation. P value = probability of chance, $^{\$}$ $P<0.05$ significant difference compared with the SHAM group, $^{\#}$ $P<0.05$ significant difference compared with the OVX group, * $P<0.05$ significant difference compared with the VD-sufficient group while, $^{\sim}$ $P<0.05$ significant difference compared with the VD-deficient group. SHAM, sham-operated group; OVX, ovariectomy; VD, vitamin D; CD36, cluster of differentiation 36 receptors; ABCA1, ATP binding cassette transporter A1.

with the coronary plaques formation [37]. Additionally, AIP, [$\log(\text{TG}/\text{HDL-C})$], is an outstanding interpreter for small dense LDL-C levels and was initially proposed to be a significant predictor of myocardial infarction [38]. It also, can roughly predict the presence and extent of coronary artery disease by non-invasive method [39]. Based on the aforementioned data, these atherogenic indices can predict the risk of CVD. Our data revealed significant higher atherogenic indices in addition to heart weights in the OVX and VD-sufficient groups when compared to SHAM group, indicating augmented atherogenic impact of this menopause-associated dyslipidaemia.

Furthermore, significant MDA increases and TAC decreases that representing free radicals stress and antioxidant deficiencies were observed in the OVX and VD-sufficient groups when compared to the SHAM. This could result from cessation of direct free radical scavenging activity and reduced expression of the antioxidants system including glutathione, glutathione peroxidase and superoxide dismutase caused by declines of both VD [40] and estrogen [41]. These data revealed menopause-associated oxidative stress state.

The co-existence of menopause-associated free radicals excess with LDL-C increase that is liable to oxidative damage [5] lead to generation of oxidized-LDL particles. These particles become no longer recognised by its native receptors LDL-R but through the scavenger receptors CD36. They are widely expressed on vascular cells including endothelial, macrophages, and smooth muscle cells in addition to, cardiac, adipocytes, and among others [6]. Unlikely

to LDL-R, CD36 receptors are not down-regulated by the excess intracellular cholesterol leading to massive vascular cholesterol accumulation and lipotoxicity [7]. This goes hands with our findings that revealed significant upregulation in the aortic CD36 expressions and aortic TC contents in the OVX and VD-sufficient groups when compared to the SHAM one.

Afterwards, the increased aortic TC accumulation triggers an inflammatory response in the arterial intima that proceeds to atherosclerosis process with involvement of JNK signaling molecules [42]. Vast of evidences links JNK activity to vascular atherosclerotic disease since JNK was discovered to be highly active in human atheromatous plaques [43], and commonly concentrates in the vascular smooth muscle cells and macrophages [44]. Additionally, JNK activation accelerates cytokine-induced expression of vascular and intercellular adhesion molecules-1, key elements in vascular monocyte recruitment and the subsequent exaggerated inflammatory reactions [45]. Our results revealed an observed increase in the aortic JNK activity in the OVX and VD-sufficient group compared to the SHAM. This could be explained by withdrawal of estrogen's JNK-suppressor effect [46] and menopause-associated oxidative stress, dyslipidaemia, and inflammatory cytokines that are known stressors activating JNK [42].

Thus, the likelihood of discovering agent that has lipid-lowering, antioxidant, and JNK inhibitor effects with subsequent reduction in the vascular CD36 receptors and cholesterol accumulation, appeared promising to combat atherosclerotic risk.

VD is a multifunction vitamin essential for cardiovascular health [17]. So, in order to explore the effect of VD status on either prevention or acceleration of atherosclerotic risks, VD-deficient-HFD (25 IU VD/ Kg diet) and VD-replete HFD (10 000 IU VD/ Kg diet) were used. They were chosen to attain target 25-OH VD levels of insufficiency and sufficiency [20, 22, 33]. VD dietary intervention led to corresponding difference in the serum 25-OH VD levels, with VD-deficient group exhibited the lowest serum 25-OH VD levels and VD-replete group showed the highest serum 25-OH VD levels versus other groups; SHAM, OVX, and VD-sufficient group. Moreover, we have found that VD-deficient HFD resulted in further aggravation in dyslipidaemia, atherogenic indices, oxidative stress, aortic CD36 receptors expression, and aortic TC content and JNK activity when compared to VD-sufficient HFD. On the contrary, these parameters were significantly improved by VD-replete diet. Multivariate linear regression analysis of the association between serum 25-OH VD levels and atherogenic indices revealed an inverse significant association with AIP and AIP is the most predictor for serum VD levels.

Our findings were in agreement with retrospective studies linking VDD to atherogenic lipid profile [11, 47]. Moreover, Vitezova et al. reported high 25-OH VD levels are concomitant to higher HDL-C levels in elderly [48]. Additionally, Mahmood et al. recently published high VD levels are associated with a favourable lipid profile in patients with cardio-metabolic syndrome [49]. In contrary to our findings, Ponda et al. have reported no improvement in the lipid profile [50] but this could be attributed to the short-term (8 weeks) VD supplementation.

VD-growing up effect on the HDL-C levels was suggested as a mechanism for its lipid lowering effect [11]. To explore that, we assessed the vascular expression of ABCA1 transporter responsible

for free cholesterol transfer from peripheral tissues to HDL-C and essential for nascent HDL-C formation [12]. We found VD-replete diet caused significant abundant aortic ABCA1 expression with subsequently higher serum HDL-C when compared to the other groups. This helps cholesterol clearance from vascular wall. Also, HDL-C has antioxidant [51], anti-inflammatory [11], and endothelial protective [52] effects.

Furthermore, the hypolipidemic effect of VD-replete HFD also, involves reduction in saturated fatty acid absorption from the gut due to formation of calcium-fatty insoluble complexes [53], increasing the conversion of cholesterol to bile acids that excreted in bile [54]. Additionally, VD induces lipoprotein lipases activity [55], and helps free fatty acids transport into mitochondrion for oxidation [56]. Accordingly, VD through its HDL-C growing effect and TGs and LDL-C lowering effect appeared promising agent in lipid-lowering strategy.

The antioxidant properties of VD [40], was able to restore the redox balance of ovariectomized rats. That was evidenced by significant increase in TAS and decrease in MDA levels in VD-replete group when compared to other groups. With improved menopause-associated oxidative stress and dyslipidaemia, known stressors for JNK activation [42], arrested JNK activity was stemmed in the VD-replete group. Inhibition of JNK activity causes reduction in CD36 receptors expression [9] and subsequently attenuates aortic TC content. Our findings were in agreement with Oh et al. that recently reported deletion of JNK prevents VDD-induced atherosclerosis in mice in part due to decreased CD36 expression [9]. Thus, VD-repletion state improved menopause-associated risks for atherogenesis and hence decreased prevalence of atherosclerotic CVD.

To interpret these biochemical risks at the cellular level, histopathological examination of the vascular wall was recommended in addition to arterial blood pressure measurement. Also, further studies would be conducive to assess possible other molecular mechanisms of VD.

Conclusion

Alarming levels of VDD among ovariectomized rats were observed. VD-deficient state aggravated menopause-associated dyslipidaemia and the increased atherogenic indices while, VD repletion state improved it. HDL-C growing up and LDL-C lowering effects in addition to improvement of redox status and JNK inhibition were among the possible mechanisms. Therefore, we recommend VD as an alternative strategy or supplements during menopause, being a natural, widely available, easily applicable and cost-effective agent. Furthermore, VD screening tests for postmenopausal women might be helpful to detect VDD earlier and allowing healthy menopause transition.

Author Contribution Statement

All authors have participated sufficiently in the conception and design of this work or the analysis and interpretation of the data, as well as the writing and reviewing of the final version of the manuscript.

Acknowledgment

We gratefully thank the technical and administrative staff at the physiology and Biochemistry departments, Faculty of Medicine, Benha University, for their assistance and support.

Conflict of Interest

The authors declare that they have no conflict of interest.

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