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Original Research

Modulation of S100 and smooth muscle actin-α immunoreactivity in the wall of aorta after vitamin D administration in rats with high fat diet

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Abstract: High fat diet is a risk factor for the development of atherosclerosis. Hence, research studies are important to understand the cellular and molecular mechanisms of atherosclerosis pathogenesis. The current study was conducted to evaluate the role of vitamin D in modulation of aortic histopathological, immunohistochemical alterations and biochemical changes induced by high fat diet in male albino rats. Forty adult rats were divided into three major groups; group I (control), group II (High fat diet) and group III (High fat diet with vitamin D). At the end of the experiment, blood cholesterol and triglycerides were determined. Aortic arches specimens were collected for histopathological study and immunohistochemical staining. Aorta of high fat diet group showed intimal thickening with vacuolated endothelial cells. The tunica media showed areas of fibrosis and irregular vacuolated smooth muscle cells. Many inflammatory cells were detected in the tunica adventitia. Significant reduction in area percentage of smooth muscle actin-α (SMA-α) immunoreactivity and increase in number of S100 positive dendritic cells (DCs) with significant increase in serum cholesterol and triglycerides were also detected. Concomitant vitamin D supplementation, with high fat diet, showed amelioration in histopathological aortic changes with significant increase in SMA-α immunoreactivity and decrease in S100 positive (DCs). However, serum cholesterol and triglyceride showed non-significant decrease after vitamin D supplementation. In conclusion, vitamin D administration ameliorates aortic wall histoopathological changes induced by high fat diet most probably through local modulation of S100 and SMA-α immunoreactivity. Hence, vitamin D could be suggested as a protective agent against aortic atherosclerotic changes.

Key words: Dendritic cells; High fat diet; Microscope; S100 protein; SMA- α; Vitamin D.

Introduction

Atherosclerosis was reported to be developed as a result of chronic inflammatory reaction in the intima of large arteries. Low Density Lipoprotein (LDL) deposition in the arterial wall stimulates chronic inflammatory reaction with production of chemical mediators and recruitment of macrophages and T-lymphocytes (1,2,3). Vascular smooth muscle cells (SMCs) are considered important factor in atheroma formation (4).

Dendritic cells (DCs) were reported to play role in the development of atherosclerosis which are antigen presenting cells originating from bone marrow and take essential role in both innate and adaptive immunity (5,6). Some studies found a network of DCs in the wall of normal arteries, elevating the question if there is a role for DCs in the development of inflammation in this tissue (7). Many studies showed decreased blood DCs number in patients with atherosclerosis. Other investigators attributed this to the recruitment of DCs to the site of inflammation where they are activated by altered antigens of apoptotic cells and oxidized LDL (8,9,10,11). They engulf, process antigens, and present them through surface major histocompatibility complex -class I (MHC-I) or MHC-II to T lymphocytes (5).

Vitamin D was reported to have a role in modulation of cell growth, neuromuscular and immune function, and reduction of inflammation (1,3).Moreover, most tissues have vitamin D receptors (VDRs), and many genes encoding proteins that regulate cell proliferation, differentiation, and apoptosis are modulated in part by vitamin D (12).

Supplementation with 800–1000 IU of vitamin D per day was reported to be needed, in the absence of adequate sun exposure, to achieve the maximum beneficial effects of vitamin D for health in children and adults (13). Vitamin D deficiency is a common health problem and about 1 billion persons suffer from vitamin D deficiency (14). Many studies revealed an association between decreased vitamin D level and cardiovascular diseases (CVD) including subclinical carotid atherosclerosis and coronary artery diseases (15,16,17). The roles of vitamin D at cellular and molecular levels was supported by the previous observational and some prospective data that reported its protective role in immunity and cardiovascular disorders (18). Moreover, it was found that the anti-artherogenic effect of vitamin D may be related to its anti- inflammatory action as vitamin D modulates both the innate and adaptive immunity and plays important role in T-cell and macrophage differentiation, activation and function (19,20,21,22). It has also been found that vitamin D inhibits DCs differentiation,

maturation, activation and function (23).

On the other hands the oxidative micro-environment associated with chronic inflammation was reported to result in vitamin D degradation and inhibits the essential enzymes for vitamin D synthesis (24). This debate raises the question is there a beneficial effect of vitamin D supplementation in preventing or slowing atherosclerosis. So, the current study was conducted to evaluate the role of vitamin D in modulation of aortic histopathological and immunohistochemical alterations induced by high fat diet beside serum lipid profile changes in male albino rats.

Materials and Methods

Experimental animals

Forty adult male albino rats (6 months old & 220 ± 20 gm) were obtained from the Egyptian Organization for Biological Products and Vaccines. Rats were kept in individual polyethylene cages with stainless-steel tops at the Animal House, Faculty of Medicine, Zagazig University. Rats were subjected to controlled conditions of temperature (25 ± 2ºC) and allowed free access to diet and water ad libitum. To prevent cutaneous synthesis of vitamin D, lights were shielded with UV sleeves. The experiment was carried out in compliance with the "Guide of the Care and Use of Laboratory Animals" (25). The experimental protocol was approved by the ethical committee of the Faculty of Medicine, Zagazig University.

One week after acclimatization rats were randomly divided into three major: *Group I (control)*: twenty rats were divided into 2 equal subgroups, ten rats each: Subgroup Ia *(Negative control):* were received regular diet (12% as fat) and water for 6 months. Subgroup Ib (*Vitamin D):* as rats of subgroup Ia and were received vitamin D in a dose of100 IU vitamin D/ kg for 2 weeks (26). *Group II* (*High Fat Diet)*: ten rats were fed high-fat diet for 6 months. The high-fat diet were prepared in Faculty of Agriculture, Zagazig University contained 210 kcal/100 g/day; 58% fat, 25% protein and 17% carbohydrate as a percentage of total kcal for 6 months (27). The chow was presented in the powder form, and admixed with 30% - 50% melted animal abdominal fat until the mixture became dough. The dough was put into blocks, dried and used for feeding the rats (28). Ca intake was chosen as 50% of the minimum intake for maximum Ca retention (0.2 % Ca) to avoid suppression of the vitamin D effect by high ca intake (29). *Group III* (*High Fat Diet + vitamin D)*: ten rats received high fat diet as group II simultaneously with administration of vitamin D in a dose of 100 IU vitamin D/ kg for 6 months (26). The used vitamin D (as cholecalciferol) named "Vi D3", the container of 10 ml contained 4500 IU so dissolved in 300ml of olive oil (so, each 1 ml of the formed mixture contained about 15 IU) and the rats were weighted every week and given their required doses. e.g., rats weighted 250 gm were receive 25 IU/ day (~2ml of the prepared mixture). During the study period, the animals were weighed once a week and body length (nose-to-anus length) was measured to calculate body mass index (BMI) (= body weight (g)/length^2 (cm^2) to estimate obesity (30).

Light microscope study

At the end of the experiment, rats were anaesthetized using ether inhalation and aortic arch specimen were taken and processed for light microscopy. The specimens were fixed in 10% formal saline for 12 hours to prepare paraffin blocks. Sections (5 µm thick) were prepared and stained by hematoxylin and eosin stain (H&E) (31). Immunohistochemistry was performed by Ventana 320 ES automated immunostainer (Ventana Medical Systems, Tucson, AZ) using an indirect, biotin-free system kit (Ultra View Universal DAB Detection Kit Ventana Medical Systems, Germany, and Catalog Number: 760-500) according to the manufacture. Briefly, paraffin sections were mounted on charged slides. Sections were deparaffinized by incubation in xylene followed by descending concentrations of ethanol. Then, 3% hydrogen peroxide was used to block endogenous peroxidase. Sections were microwaved in 10 mM citrate buffer pH 6.0 for antigen retrieval. Blocking nonspecific staining was performed using 1% bovine serum albumin. Sections then were incubated with the primary antibodies overnight at 4°C, (anti-SMA-α, Ventana Medical Systems, Tucson, AZ, USA, Catalog Number: 760-2833 and anti-S100 protein, Ventana Medical Systems, USA, Catalog Number: 790-2914). Both used primary antibodies were mouse monoclonal IgG antibodies, with cytoplasmic immunohistochemical reaction. Sections were counterstained by Mayer's hematoxylin. Each run, a section of appendix and a section of melanoma were considered positive controls for SMA-α and S100 protein respectively, and phosphate buffered saline (PBS) was used instead of the primary antibody as negative control (32).

Biochemical study

Blood samples were collected via retro-orbital sinus puncture and centrifuged at 14000 rpm at 4°C for 10 min to obtain plasma. Plasma was delivered to the Clinical Laboratory at Zagazig University for analysis of total cholesterol and triglyceride levels in all groups and data were statistically analyzed.

Morphometric study

The image analyzer computer system Leica Qwin 500 at the Research Unit, Faculty of Medicine, Taibah University, Al Medina Al Monawarrah, KSA was used to evaluate the following parameters. The mean area percentage of SMA-α- positive vascular SMCs was measured at a magnification X400 for in 10 consecutive fields from each rat of all groups. The number of S100 protein immunoreactive DCs were counted in five random fields (x400) per slide and the mean number were calculated for each rat (33). The color detect menu in relation to a standard measuring frame was used for measurements.

Statistical analysis

Data for all groups were expressed as mean ±standard deviation (X±SD). Data were analyzed using SPSS program version 15 (Chicago, USA; ''http// WWW.SPSS.com''). One-way analysis of variance (ANOVA) with Fisher multiple comparison test was used for statistical comparison between different means. A p value less 0.05 was considered statis-

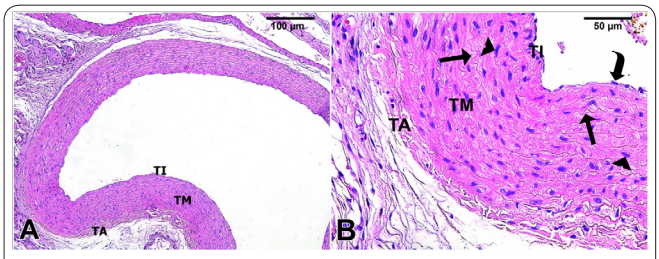

Figure 1. Photomicrographs of the aorta of control group. A&B: showing tunica intima (TI) with flat endothelial cells (curved arrow), tunica media (TM) with regular parallel, continuous and wavy elastic laminae (arrows) and regularly arranged SMCs with oval nuclei (arrow heads). Tunica adventitia (TA) with loose connective tissue is observed. (H&E stain, scale bar: A=100 µm and B= 50 µm).

tically significant.

Results

Histopathological results

Light microscopic examination of the aorta of control subgroups Ia and Ib showed similar results thus, subgroup Ia was chosen as the control group in the interpretation of the results. Aorta sections stained with H&E of control group showed the wall of the aorta formed of; tuinca intima with its endothelial cells, tunica media with regular parallel, continuous and wavy elastic laminae and regularly arranged spindle smooth muscle cells

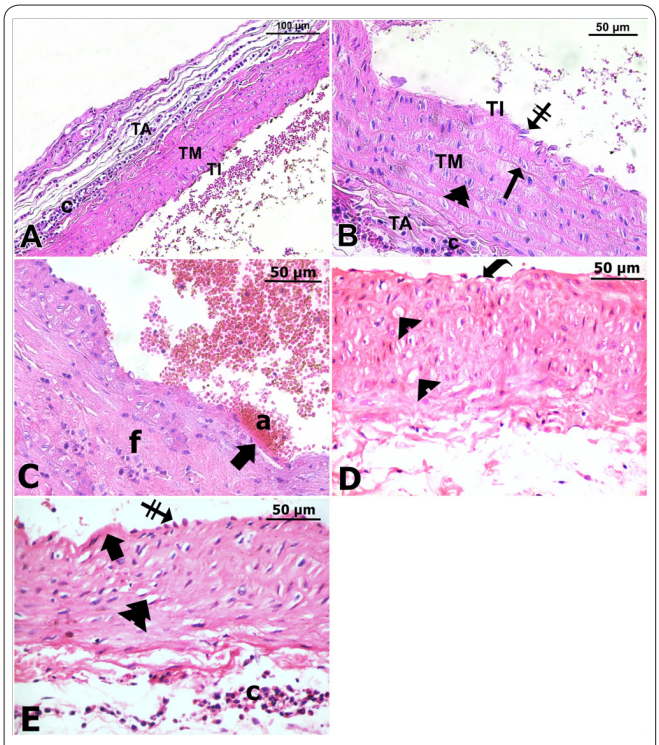

Figure 2. Photomicrographs of the aorta of high fat diet group. A-E: showing tunica intima (TI) with darkly stained endothelial cells and bulging nuclei (crossed arrow). Tunica media (TM) showing relatively straight elastic fibers (arrow) and irregular SMCs with darkly stained nuclei (double arrowheads). Sticky endothelium with RBCs adhesion (a), intimal thickening (thick arrow) and endothelial vacuolations (curved arrow) are observed. Tunica media (TM) appears with areas of fibrosis (f) and multiple vacuolations (arrow heads). Tunica adventitia (TA) shows many cellular infiltrations (c). (H&E stain, scale bar: A = 100 µm and B-E = 50 µm).

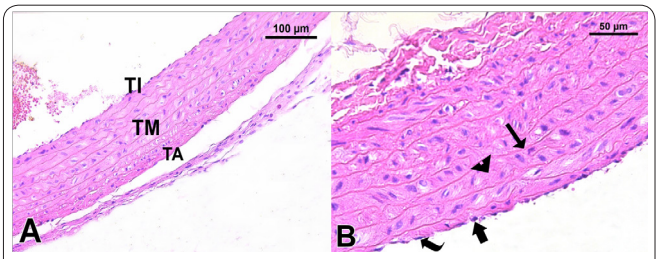

Figure 3. Photomicrographs of the aorta of high fat diet and vitamin D group. A&B: Tuinca intima (TI) shows endothelial cells most of them are flat cells (curved arrow) with few are with bulging nuclei (thick arrow). The tunica media (TM) shows regular, parallel and straight elastic laminae (arrows) with some vacuolated smooth muscle cells (arrow heads). Tunica adventitia (TA) with loose connective tissue is observed. (H&E stain, scale bar: A= 100 µm and B = 50 µm).

(SMCs) with oval nuclei and tunica adventitia consisted of loose connective tissue (Figs.1 A&B).

In the high fat diet group, some endothelial cells of tunica intima appeared with vacuolations and others with darkly stained bulging nuclei with intimal thickening. RBCs adhesion to a sticky endothelium was observed. The tunica media appeared with multiple areas of vacuolations and relatively straight elastic fibers. Some irregular SMCs with darkly stained nuclei and areas of fibrosis were observed. Many inflammatory cellular infiltrations were detected in the tunica adventitia (Figs.2 A-E).

In the high fat diet and vitamin D group, the tunica intima appeared with endothelial cells most of them were flat while few were with bulging nuclei. The tunica media appeared with regularly arranged parallel, continuous and straight elastic laminae with presence of some pale stained SMCs. Tunica adventitia appeared with loose connective tissue (Figs. 3 A&B).

Sections of aorta stained immunohistochemically with anti- SMA- α antibodies showed strong immunoreaction in smooth muscle cells (SMCs) of tunica media of control group (Figs.4 A&B). In high fat diet group (Figs.4 C&D), areas of moderate reaction and other areas with weak reaction were detected in smooth muscle cells of tunica media. However, strong reaction was observed in high fat diet and vitamin D group (Figs.4 E&F).

Aorta sections stained immunohistochemically with anti S100 protein antibody revealed immunoreactivity of dendritic cells in all the layers of the wall of aorta. The control group showed few dendritic cells (Figs.5 A&B), high fat diet group showed many dendritic cells (Figs.5 C&D) and the high fat diet and vitamin D group appeared with some dendritic cells (Figs.5 E&F).

Biochemical, anthropometric and morphometric results

The current study revealed no significant difference between GIa and GIb regarding anthropometric, biochemical and morphometric results. The body weight, BMI, blood level of cholesterol, blood level of triglyceride in both GII and GIII were significantly increased when compared to GIa. However, non significant decrease was detected in GIII compared with GII. The mean area percentage of SMA-α was significantly decreased in GII and GIII compared with GIa. However, GIII showed

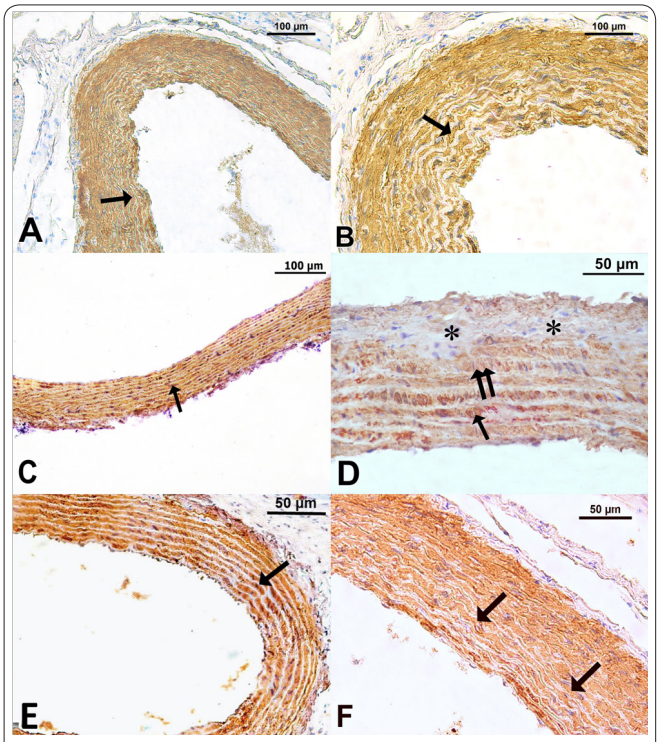

Figure 4. Photomicrographs of the aorta showing SMA- α immunoreactivity of smooth muscle cells (arrows) of tunica media which is strong in control group (A&B). Moderate immunoractivity is seen (double arrows) in high fat diet group (C&D) with areas of weak immunoreactiviry (*). Strong immunoreactivity is observed in high fat diet and vitamin D group (E&F). (Anti-SMA- α , scale bar: A-C = 100 µm & D-F = 50 nm).

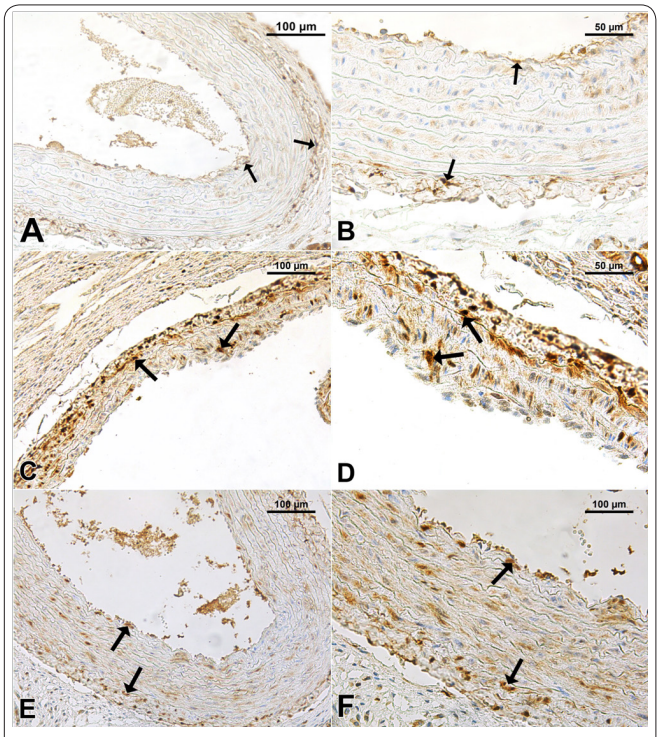

Figure 5. Photomicrographs of the aorta showing immunoreactivity of S100 protein in dendritic cells (arrows) in the wall of aorta. A&B: control group shows few dendritic cells. C&D: High fat diet group shows many dendritic cells. E&F: High fat diet and vitamin D group with some dendritic cells are observed. (Anti S100, scale bar: A, C, E, F = 100 µm & B,D = 50 µm).

significantly increased mean area percentage of SMA-α compared with GII. Significant increase in the number of S100 immunostained DCs in aortic wall was observed in GII compared with GIa. On the other hand, GIII showed significant decrease compared with that of GII although their number was still significantly increased compared with GIa (Table 1).

Discussion

The increasing prevalence of atherosclerosis in developing countries is attributed to the progressive shifts to the westernized high fat diet (34). In the current study all rats of high fat diet group (GII) showed significant hypercholesterolemia and hypertriglyceridemia compared with control group. The elevated serum lipid was reported to result in endothelial cell (EC) dysfunction which was previously used as a marker of atherosclerosis (35,36,37). This may be reflected in the present work by structural changes in the wall of aorta in the form of the presence of vacuolated intimal cells and intimal thickening. That is in consistence with Rudijanto who observed that the first step of atherosclerosis formation is endothelial changes that result in endothelial cell (EC) dysfunction (38). They added that EC injury can result in decreased nitric oxide (NO) production and increased permeability to lipoprotein. Intimal thickening in the present work could be due to endothelial cells proliferation that could be supported by Miller and his colleagues who suggested that proliferation of intima, thrombosis and atherosclerosis were associated with hypertriglyceridemia and increased the low-density lipoproteins (LDL) (39). Similarly, hypertriglyceridemia in patients with type 2 diabetes is associated with carotid intimal and medial thickness (40). All of these indicated that endothelial cell injury and atherosclerosis may be accelerated by hypertriglyceridemia (35). On the other hand, atherosclerosis induced by nicotine was through EC pyroptosis (41)

This EC injury may explain the other histological alterations in the aortic wall as the endothelium was

Table 1. Statistical analysis of the biochemical, anthropometric and morphometrical parameters among the studied groups.

Variables	GIa (negative control) Mean ± SD	GIb (Vit D) Mean ± SD	GII (Fat) Mean ± SD	GIII (Fat & Vit D) Mean ± SD
Body weight (g)	376.6 ± 2.50	379.3 ± 2.26	486.0± 2.40*	425.20± 2.30*
BMI (g/cm^2)	0.61±0.01	0.63±0.03	0. 84±0.1*	0.81±0.02*
Cholesterol (mg/dl)	82.60±1.58	82.00±1.76	87.00±1.41*	86.50±1.84*
Triglyceride(mg/dl)	77.17±1.16	76.77±1.11	78.83±1.39*	80.34±4.65*
SMA-α	32.02±3.93	31.94±3.83	16.76±3.32*	26.37±3.45*#
Dendritic Cells	16.50±1.51	17.70±2.87	33.30±5.44*	24.80±2.53*#

SD, standard deviation. n = 10 in each group. * Significant difference as compared to GIa, # Significant difference as compared to GII.

reported to be a major blood vessel regulator that influences vascular constriction, dilation, SMCs proliferation, inflammation, thrombosis, and fibrinolysis (42). Thus, any EC damage can result in endothelial dysfunction that plays an important role in atherosclerosis development (36) and the inhibition of EC inflammation was reported to be a potential target to treat atherosclerosis (43, 44).

Moreover, intimal thickening in the present work could be due to inflammatory response and the cytokines and chemical mediators released from injured EC that stimulates migration and proliferation of vascular SMCs to the intima, where they produce extracellular matrix (ECM), leading to vascular wall thickness and atheroma formation (38).

Irregular vacuolated SMCs with darkly stained nuclei and areas of fibrosis were observed in the current study in high fat diet group. That could be explained by other investigators who observed that foam cells were formed as a result of phagocytosis of oxidized trapped LDL particles in the vascular wall (45). This was reflected in the present work by the presence of both intimal and medial vacuolated cells in the aorta. Subbotin attributed the deposition of lipoproteins in coronary wall to the neovascularization from adventitial vasa vasorum (46). In rabbits received cholesterol powder, the aorta developed atheromas with many foam cells (47). Similarly, in diabetes mellitus type 2, Chen and his colleagues observed foam cells in tunica media of ascending aorta (48). Also, the tunica intima of the aorta and pulmonary trunk of untreated diabetic rats showed progressive lipid deposition (49). Many inflammatory cellular infiltrations were detected in the tunica adventitia of aorta in high fat diet group. That could be explained by Hansson and Libby who considered atherosclerosis a complex inflammatory disease of the arterial wall (50).

In the high fat diet and vitamin D group (GIII), minimal histological changes of the aorta were observed compared with GII. The tuinca intima appeared with endothelial cells most of them were flat while few were with bulging nuclei. The tunica media appeared with regularly arranged parallel, continuous and straight elastic laminae with presence of few pale stained SMCs. Tunica adventitia appeared with loose connective tissue without inflammatory infiltrate. In concomitant with these findings, Liu and his colleagues found that vitamin D deficiency was significantly associated with carotid intima-media thickness (51). This association may be attributed to the ability of vitamin D to protect endothelial cells against oxidative stress and apoptosis which plays a significant role in atherosclerosis pathogenesis (52). Previous studies revealed that the endothelial cells showed vitamin D receptors (VDR) that may play a role in preventing EC dysfunction and development of atherosclerosis through different mechanisms. Vitamin D converts 25(OH) D to 1,25(OH)D through activation VDR on ECs and the expression of 1-alpha-hydroxylase and the formed 1,25(OH)D results in nitric oxide synthase transcription in ECs (53).

The improved histological structure of aortic wall in the group received vitamin D with high fat diet also could be explained by previous investigators who reported that vitamin D has been shown to affect atherogenesis through multiple mechanisms such as increases endothelial nitric oxide, decreases endothelial oxidative stress, inhibits proliferation and SMCs migration, inhibits platelet and leukocyte aggregation and adhesion, inhibits release of pro-inflammatory cytokines and modulates immune response (36,54). Moreover, SMCs were reported to possess VDR, which express 1-alpha-hydroxylase (55). Also, vitamin D was concluded to decrease vascular SMCs proliferation through inhibiting endothelin-dependent DNA synthesis and suppressing activation of cyclin-dependent kinase-2 (19). Transgenic rats as a model of vitamin D deficiency, showed aggravated atherosclerosis under a high-fat and high-cholesterol diet, when compared with control rats (56). Vitamin D also was reported to have a role in immune system regulation (57).

The current study showed that serum cholesterol and triglyceride were not affected by vitamin D supplementation as there was no significant difference between their level in rats with high fat diet (GII) and those that took vitamin D with high fat diet (GIII). In concomitant with our results, others revealed that serum lipid profile is not affected by vitamin D administration (58). Also, in a metanalysis of 12 clinical trials included 1346 patients, it was found that there is no effect of vitamin D supplementation on serum lipids (59). These findings could suggest that vitamin D may exert its effect locally on tissues rather than through decreasing serum lipid. In addition, vitamin D administration with high fat diet, in the present work, showed no significant differences in body weight and BMI when compared with high fat diet group. That is in consistence with Vanlint who observed no effect of vitamin D supplementation on prevention or treatment of obesity (60). In contrast to our findings some previous studies revealed that vitamin D deficiency is associated with hypertriglyceridemia (61). In addition, the serum cholesterol level was reported to decrease after vitamin D administration (62).

The current study showed decreased immunoreactivity to SMA-α of vascular SMCs in tunica media of GII rats compared with control. Rudijanto attributed that to the conversion of vascular SMCs from actin forming cells to fibrous tissue forming cells (38). That could be explained by Gittenberger-de Groot and his colleagues who reported that vascular SMCs migration, proliferation and extracellular matrix (ECM) production are important factors in the development of atherosclerosis (63). However, the adhesion molecules and the inflammatory cytokines expressed by vascular SMCs take essential parts in the initiation and development of atherosclerosis (4). These vascular SMCs showed decreased contractile protein and developed non-contractile or synthetic phenotype and were called dedifferentiated cells (38). On the other hand, the current study revealed that vitamin D supplementation restore this function of vascular SMCs as evident by significant increase of immunoreactivity to SMA-α of vascular SMCs in aorta of group GIII rats compared with GII.

In the present study, we used S100 protein as a marker for DCs. It is considered the most reliable and general marker of DCs in atherosclerotic plaques. It detects both immature and mature DCs (9). Aorta of control groups showed few numbers of DCs in both the intima and the adventitia. This finding is concomitant with that of previous investigators (7, 64). The number of S100

immunostained DCs was significantly increased in aorta of high fat diet group (III) compared with control group, reflecting their role in atherosclerosic changes. Similarly, Fang and his colleagues revealed large number of mature DCs in the adventitia and intima of aorta with atherosclerosis and they found a relation between their number and the progression of the disease (33). In the present work, vitamin D supplementation with high fat diet (GIII) was associated with significant decrease in S100 immunostained DCs, although their number still significantly higher than control group. That is in consistence with previous investigators who showed that vitamin D supplementation was associated with significant decrease of mature DCs in atherosclerotic plaques and in mesenteric lymph nodes and spleen (21). Others concluded that vitamin D supplementation decreased DCs number at the atherosclerotic lesion site and it also suppressed DCs maturation and modulating their function (22,23).

Haddad and his colleagues suggested that total absence of dendritic cell receptors in mice, significantly reduce inflammatory cell content within arterial plaques and supress atherosclerosis development (65). Similarly, quercetin (flavonol) was reported to effectively attenuate atherosclerosis in mice by suppressing DC activation (66).

The current study revealed that vitamin D supplementation ameliorates histolopathological alterations in aortic wall induced by high fat diet with increased vascular SMCs immunoreactivity to SMA-α, and decreased number of DCs. However, vitamin D supplementation has no effect on serum cholesterol or triglyceride that may suggest that vitamin D may exert its protective effect locally on aortic wall rather than through decreasing serum lipid. Hence, vitamin D supplementation may be used as a protective agent against early atherosclerosis development. Further studies are required to evaluate the role of DCs in atherosclerosis and to explore the mechanisms by which vitamin D could protect from atherosclerosis.

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Conflict of interest
There is no conflict of interest.

Author contribution
Author 1 was responsible for laboratory work, data analysis and writing. Author 2 was responsible for writing, interpretation of figures and publication. Author 3 was responsible for histological preparations and photographing histological slides. Author 4 was responsible for writing the protocol of the research.

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