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Analysis of the lipid profile, activity of antioxidant enzymes and inflammatory molecules after vitamin D supplementation in an experimental model of diet-induced atherosclerosis

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ABSTRACT

Low levels of vitamin D increase the risk of cardiovascular disease. The objective of this study is to verify the effects of vitamin D in an experimental model of rabbits fed a diet rich in lard / sucrose / cholesterol (LSC). The cholesterol-fed rabbit model is notable for rapid development of aortic lesions and low cost for maintenance, being a typical diet for induction of atherosclerosis, supplementation of 0.5% to 4% of cholesterol in about 8 to 16 weeks. Considering a possible protective effect of vitamin D on the cardiovascular and hypercholesterolemic/hyperglycemic diet, as an important risk factor, in this study, we examined the action of vitamin D in an experimental model of rabbits fed a diet plus cholesterol, lard and sucrose, analyzing inflammatory molecules such as ICAM-1, MCP-1 and e-NO in the aorta; activity of antioxidant enzymes such as superoxide Dismutase (SOD), Catalase (CAT), as well as lipid profile parameters. The levels of total cholesterol, Triglycerides and VLDL-c showed a considerable decrease when we compare the results of the 12th and 24th week. The number of animals was the limiting factor of our study, further analysis should be made to understand the mechanism of vitamin D in experimental atherosclerosis.

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Introduction

Cardiovascular diseases (CVD) are the leading causes of morbidity and mortality worldwide. According to the data presented by the World Health Organization (WHO), 17.7 million people have been died in 2015 because of CVD, which accounts for 30% of the total registered globally. The WHO estimates that by 2030, approximately 23.6 million people in the world die by CVD[1]. In Brazil, the CVD are responsible for about 20% of all deaths in individuals over 30 years[2].

One of the manifestations which leads to CVD is atherosclerosis, which are the atheroma plates, characterized by focal accumulation of lipids, carbohydrates, blood, fibrous tissue and calcium deposits on artery intima layer. The formation of atheroma plates can occur due to chronic inflammation of the arterial wall endothelium injury initiated, where the accumulation of these components can limit blood flow, causing stenosis[3,4]. The atherogenesis therefore characterized by the

development of atheromatous plaques, on the inner surface of the arterial walls, where the crystals of cholesterol, along with cell proliferation may protrude from the arterial lumen, causing flow reduction blood, leading to complete occlusion of the vessel[5].

Among the factors that lead to the formation of the atheroma plates, include advanced age, hypertension, hypercholesterolemia, smoking, obesity, radiotherapy of head and neck, coronary artery disease, genetic inheritance, sedentary lifestyle, stress and Diabetes mellitus[6,7].

During the atherogenesis process, adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are secreted as well as monocyte chemoattractant protein-1 (MCP-1), which is a molecule of monocyte chemoattractant monocytes. ICAM-1 is one of adhesion molecules that make up the family of immunoglobulins, and which participates in the inflammatory process caused by atherosclerosis. The molecules ICAM-1, act promoting exclusion, adhesion and diapedesis of circulating monocytes in light of the endothelium to the subendothelial space[8]. MCP-1's recruiter of monocytes and is involved in inflammatory processes stemming from atherosclerosis and diabetes. At the beginning of the atherogenesis, MCP-1 is the link between lipid peroxidation and the recruitment of macrophages, being then considered the key molecule in regulating phagocytosis of the molecules of LDL-ox and foamy cell formation. In addition to participating actively in the early stages of the formation of atherosclerotic plaque, MCP-1 also participates actively in the process of destabilization of plate, because it induces the production of matrix Metalloproteinase which favors the break[9].

Together, this series of changes in the endothelium, leading to the pro-inflammatory phenotype. There is also an increase of reactive oxygen species (ROS), cytokines and growth factors that lead to the proliferation of smooth muscle cells and the formation of foamy cells[10,11,12,13]. Oxidative stress is a condition in which excess formation of highly reactive molecules, such as ROS and reactive nitrogen species, cause an unbalance natural antioxidant defense mechanism, resulting in damage oxidative[11,12]. The antioxidant enzymes such as superoxide Dismutase, Catalase and Glutathione Peroxidase are part of the enzymatic defense system and work on reducing the deleterious effects caused by the EROs[14]. Oxidative stress is associated with the pathogenesis of atherosclerosis by oxidation of lipids that settle in the subendothelial space. Oxidized lipids promote vascular damage, because they induce the expression of Pro-Inflammatory Cytokines, consequently increasing the ROS in injury. Studies show that in the early stages of atherogenesis, the activity of antioxidant enzymes of SOD and CAT are higher, however, with the progression of atherosclerosis enzyme activities tends to decrease[14,15].

Low levels of vitamin D are associated with CVD, obesity, hypertension and dyslipidemias[16]. Studies report that adequate amounts of vitamin D are involved in reducing cardiovascular risk factors such as hypertension and the components of the metabolic syndrome, as well as to your disability seems to be involved in the process of arterial aging and atherogenesis[17,18]. There is a strong association between low concentrations of 25 (OH) D and coronary events, congestive heart failure, stroke or cardiovascular mortality. It should be noted that there is a relationship between vitamin D deficiency and the process of atherosclerosis[19,20,21] (Figure 1). The Hypovitaminosis D was recognized as an important risk factor for many diseases, given this alarming picture epidemiological studies have demonstrated that a portion of the world's population, regardless of geographic location, ethnicity and age present low levels of vitamin D. In Brazil, approximately 60% of the adolescents, 40% to 58% of young adults and 42% to 83% of the elderly present low levels of vitamin D in the body[22]. In this picture, the interest in prevention and cure of diseases through vitamins supplementation increases every day. In this sense, the vitamin D presents multiple effects in biological processes, not only in the regulation of calciumphosphorus metabolism, but also in cell proliferation and differentiation, in the regulation of apoptosis and immune system[23]. Vitamin D is a fat soluble in the family of steroid hormones and can be obtained by direct synthesis in the skin by ultraviolet light B or by feeding. In food, it can be found in plant sources such as vitamin D2 (ergocalciferol) that includes a wide variety of mushrooms, such as Shiitake, Enoki and Portobello, or animal sources, such as vitamin D3 (cholecalciferol) present in milk, eggs and fish such as salmon, sardines and tuna[23,24].

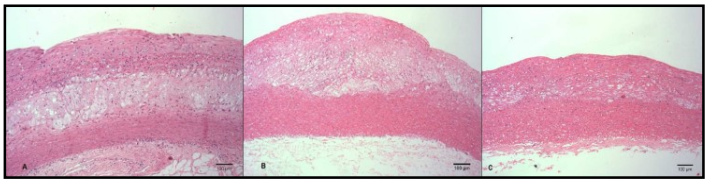

Figure 1. Photomicrograph of the histological section of the aorta, in the (A) Arch, (B) Thoracic and (C) Abdominal portions. HE staining.

In the skin, vitamin D is synthesized in the basal layer of the epidermis, prickly and as it is in lipid bilayer membranes which is located the precursor molecule, the 7-naturally occurring (7-DHC), through the photochemical cleavage of 7-naturally occurring by ultraviolet B[25]. Independent of the absorption by the intestines through the ingestion of vitamin supplements or diet or by synthesis in the skin, vitamin D is carried to the liver with the aid of plasma glycoprotein BPD (vitamin D binding protein) which is the binding of protein Vitamin D[23,24].

In the liver, vitamin D3 undergoes hydroxylation in the position of the first carbon C-25, making it 25-hydroxyvitamin D or calcidiol (25 (OH) D3) by the enzyme microsomal 25-hydroxylase, also called CYP2R1, located in the cytochrome P450. The 25 (OH) D3 is the main circulating metabolite, and will be carried by the BPD to the kidneys, where it will be hydroxylating by the enzyme 1 α-hydroxylase (CYP27B1) in proximal tubules, leading to the active metabolite 1.25 (OH2) D3 also known as calcitriol[14]. Between the different models and experimental designs for study of atherosclerosis, the rabbit is a

good model for this study, due to metabolism of lipoproteins are more like those of humans[26].

The cholesterol-fed rabbit model is notable for rapid development of aortic lesions and low cost for maintenance, being a typical diet for induction of atherosclerosis, supplementation of 0.5% to 4% of cholesterol in about 8 to 16 weeks. Under these conditions, the rabbits become hypercholesterolemic quickly (with plasma cholesterol > 1,000 mg/dl) and the resulting lesions consist primarily of macrophages derived foamy cell[27,28,29,30]. Considering a possible protective effect of vitamin D on the cardiovascular and hypercholesterolemic/hyperglycemic diet, as an important risk factor, in this study, we examined the action of vitamin D in an experimental model of rabbits fed a diet plus cholesterol, lard and sucrose, analyzing inflammatory and oxidative stress parameters in the aorta.

Abbreviations

CVD: Cardiovascular diseases; **ICAM-1**: Intracellular Adhesion Molecule-1; **LSC**: Lard / Sucrose / Cholesterol; **MCP-1**: Monocyte Chemoattractant Protein-1; **RNS**: reactive nitrogen species; **ROS**: reactive oxygen species; **VCAM-1**: vascular cell adhesion molecule-1

Material and methods

All the procedures adopted in this study were approved by the Research Ethics Committee of the University Federal of the São Paulo. We use 20 New Zealand breed rabbits, with 3 months of age and approximately 3 kg from the Rabbit Center – Criex. The experimental period lasted 24 weeks and during this period, the animals remained in the Vivarium of University Federal of the São Paulo, in individual cages under controlled conditions of temperature (22 + 2° C), light/dark cycle of 12 hours and exhaustion, receiving water ad libitum and standard ration during the acclimatization period. After the acclimatization period, the animals were divided into four groups (n = 5) and received LSC and standard ration according to the experimental group. LSC food was increased by 10% of lard, 40% sucrose and cholesterol powder (Merck®) at concentrations of 0.5% during the first 12 weeks and 0.1% in the last 12 weeks, we administer vitamin D 1000 IU daily dose to animals for 12 weeks. The groups were constituted as follows: GI: LSC + vehicle; GII: LSC + Vit. (D); GIII: LSC/Standard + Vit. (D); GIV: LSC/standard + vehicle.

Completed the trial period, the animals were euthanized with 35 mg/kg of Ketamine hydrochloride (Ketalar®, Parke-Davis, USA) and 5 mg/kg xylazine hydrochloride (Rompum®, Bayer, USA) via intraperitoneal. We performed the asepsis of the region and made a median incision thoracic abdomen. The blood was collected by cardiac puncture for analysis of biochemical Col. Total, HDL-c, VLDL-c, TG, insulin, glucose, and activity of antioxidant enzymes, SOD and CAT. Remove the aorta of the arc to the iliac bifurcation for Histopathological Analysis, histomorphometry, and expression of ICAM-1 biomarkers, MCP-1 and us, by Immunohistochemistry.

The aorta was subjected to stained of Sudam III to show the lipid content and were analyzed with respect to the presence of fatty plaques in your extension. For Histopathological Analysis of the samples, the fragments were separated in arch, thoracic and abdominal and were stained by hematoxylin and eosin techniques for analysis and histological classification and Morphometry, Von Kossa to visualize the presence of calcium and Picro-sirius for analysis of fibrosis under polarized light. Macro and micro information they were associated with and classified according to the criteria established by Stary, in accordance with the guidelines established by the American Hearth Association[31]. The images were captured with the aid of a video camera connected to a microscope Olympus BX-40, digitized and transmitted to a computer. Using a program to Morphometry, IMAGE TOLL FOR WINDOWS 3.0 VS (The University of Texas Health Science Center in San Antonio UTHSCSA, USA), we measured the largest height of the plates (µm), the intima and media areas (mm²) and the calculation of the intimate relationship (I/M). For analysis of fibrosis and calcification, the slides were scanned and quantified using Image software J.

For Immunohistochemistry analysis, we use the ICAM-1 antibodies (1:100) (Santa Cruz Biotechnology, G-5, sc8439); anti-MCP-1 (1:100) (Dako); and-in (1:100); Insulin (1:5) (Lot 2491449-Merck-Millipore). The antigenic recovery was accomplished with the use of Steamer (Arno, São Paulo, SP) in citrate buffer pH 6.0 for 40 minutes. The primary antibodies were hatched in BSA solution to 1% in PBS, pH 7.4 buffer in wet darkroom to 4ºc for 24 hours. The fabrics were covered with secondary biotinylated Polyclonal Antibody (Polyclonal Rabbit Anti mouse Immunoglobulins/Biotinylated-E0354, Dako, Denmark), the slides were rinsed with PBS and added the solution of streptavidin peroxidase complex (strept ABComplex, Dako, Denmark) for 30 min for revelation of the blades, use the chromogen DAB ´ 3.3-diaminobenzidine (Sigma Aldrich Co. USA) and 1% hydrogen peroxide in PBS, pH 7.4 buffer for 5 minutes. Hematoxylin Harris was used to against staining, the slides were mounted with Entellan resin (MerckGermany). For analysis of the biochemical parameters, defrosted samples at room temperature for 2 hours and use the commercial Kit Labtest colorimetric enzymatic test® according to the manufacturer's information. The plates were read by spectrophotometer at a wavelength of 550 nm.

The concentrations of blood glucose (mg/dL) were measured with the aid of Roche Accu-Chek® blood glucose monitoring system using the test strips according to the manufacturer's manual. SOD and CAT were analyzed through the Spectrophotometric method according to the protocols established by Mc Cord and Fridovich (1969)[32] and Adamo et al., (1989)[33] The readings were held in spectrophotometer at a wavelength of 550nm at 25° c.

Statistical Analysis

We use the software GraphPad Prism 4.0 and the results were expressed as mean and standard deviation. For non-parametric analysis of the data, we used the Kruskall-Wallis test and, for

individualization, the test of Dunn. For frequency distribution analysis using Fischer's exact test. In all the analyses was adopted the value of < 0.05 p to statistical significance. Unfortunately, we lost four animals during the trial period, which interferes directly in our statistical results.

Results

The animals were weighed during the periods 0, 12 and 24 weeks (Table 1) and all gained weight during the trial period independent of the group. The data of the serum lipid profile dosages, glucose and insulin are presented in Table 2. The levels of total cholesterol, Triglycerides and VLDL-c showed a considerable decrease when we compare the results of the 12th and 24th week. Triglycerides and VLDL-c were considered significant among groups GIII < GII. The values of HDL-c, insulin and Glucose were not significant between the groups, however we found that insulin levels were higher in the GI group. In our experimental model no animal developed hyperglycemia.

The macro and microscopic analysis of sections of the aortas of all groups showed lipid content throughout your portion and correspond to atherosclerotic lesions resulting from high-fat diet (Table 4). We observe that the groups GI and GII showed greater commitment of the aorta with when compared to groups GIII and GIV, Histopathological Analysis showed that the groups GI and GII were more cell phones, while the groups GIII and GIV presented fibrous characteristic (Figure 1). We observed significant difference regarding the percentage of plate in the aorta, and GII > GIII (Table 5). The parsed data of intimate relationship x average showed that even in the absence of significant difference, the Group presented a less intimate relationship GIII x average when compared to other groups (Table 6). Von Kossa staining showed significant difference only in the abdominal area between the groups GII, GIII < regarding areas of fibrosis, we see greater commitment in the aortic arch, mainly in GIII and GIV (Tables 6, 7; Figures 2, 3).

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Table 1. Evolution of the body mass gain of the groups that received LSC and vitamin D diet.

Weeks	GI (n=4)	GII (n=5)	GIII (n=4)	GIV (n=3)	p(GIxGIIxGIIIxGIV)[a]
0	2,633 ± 0,1686	2,608 ± 0,1983	2,668 ± 0,1887	2,614 ± 0,1630	0,9275
12	3,038 ± 0,2438	2,947 ± 0,2165	3,262 ± 0,4959	3,288 ± 0,5905	0,6878
24	3,333 ± 0,0590	3,360 ± 0,4624	3,813 ± 0,8219	4,303 ± 0,4827	0,0990

Data expressed as mean and standard deviation. GI and GII - diet LSC 0.5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. a Statistical test - Kruskal-Wallis and Dunn's test.

Table 2. Serum parameters of the lipid profile, glycemia and insulin of the groups that received LSC and vitamin D diet

Parameters	Weeks	GI (n=4)	GII (n=5)	GIII (n=4)	GIV (n=3)	p(GIxGIIxGIIIxGIV)[a]
Total Chol. (mg/dL)	0	71,25 ± 11,6	68 ± 12,7	56,33 ± 4,9	58,8 ± 5,2	0,0971
	12	689,8 ± 187,5	860,8 ± 135,7	667,0 ± 123,6	687,0 ± 108,8	0,1208
	24	420,3 ± 128,1	363,4 ± 119,0	162,0 ± 132,1	149,3 ± 135,9	0,0551
Triglycerides (mg/dL)	0	118,3 ± 6,3	124,3 ± 26,0	110,7 ± 5,8	113,2 ± 12,9	0,6908
	12	342,5 ± 181,9	409,8 ±285,2	162,0 ± 41,7	254,8 ± 195,8	0,0534
	24	171,8 ± 58,3	202 ± 78,8	62,5 ± 12,6	74,33 ± 23,6	0,0096*
VLDL-c (mg/dL)	0	23,65 ± 1,26	15,55 ± 3,25	13,84 ± 0,73	22,64 ± 2,59	0,0078
	12	68,5 ± 36,38	81,97 ± 57,04	32,4 ± 8,34	50,96 ± 39,17	0,0534
	24	34,35 ± 11,67	40,4 ± 15,77	12,5 ± 2,52	14,87 ± 4,72	0,0096*
HDL	0	51,75 ± 1,89	52,17 ± 2,64	51,67 ± 8,32	67,6 ± 39,47	0,9053

(mg/dL)	12	142,3 ± 27,33	113,5 ± 17,67	101 ± 35,94	98,2 ± 6,22	0,0522
	24	63,25 ± 15,50	61,6 ± 9,09	58,25 ± 4,85	56,67 ± 3,51	0,8820
Glucose (mg/dL)	0	103,5 ± 6,24	110,5 ± 30,08	83,4 ± 9,76	99,6 ± 20,89	0,1051
	12	124,5 ± 23,23	108,8 ± 18,06	112,8 ± 9,96	110,8 ± 12,54	0,7279
	24	97,0 ± 4,83	92,0 ± 12,69	105,8 ± 11,09	100,3 ± 17,79	0,4461
Insulin µU/mL	0	0,98 ± 0,77	1,08 ± 1,07	0,69 ± 0,74	1,53 ± 2,21	0,6271
	12	0,85 ± 0,77	0,39 ± 0,16	0,59 ± 0,55	0,44 ± 0,22	0,5039
	24	0,65 ± 0,31	2,13 ± 2,22	0,51 ± 0,11	0,97 ± 0,63	0,8390

Data expressed in Mean and Standard Deviation. GI and GII - diet LSC 0.5 / LSC 0,1; GIII and GIV -LSC 0.5 / normal; GII and GIII - treated as vitamin D. a Statistical test - Kruskal-Wallis and Dunn's test. * p <0.05 GIII <GII.

With respect to the levels of SOD and CAT, we observe a significant difference between the groups GII and GIII, but only at time 0, with respect to trial periods, there was no statistical difference between the groups, but we found that there was a decrease of both enzymes when we compared the results of the 12th and 24th week (Table 3).

Table 3. Enzymatic activity of SOD and CAT during the experimental period

Activity enzymatic	**Week**	**GI** (n=4)	**GII** (n=5)	**GIII** (n=4)	**GIV** (n=3)	**p(GIxGIIxGIIIxGIV)[a]**
SOD Cytosolic U/mg Hb	0	8,78 ± 7,09	23,30 ± 5,67	6,95 ± 10,20	12,09 ± 7,88	0,0236*
	12	17,70 ± 9,98	13,39 ± 7,36	6,03 ± 6,84	3,16 ± 2,19	0,0831
	24	2,57 ± 0,98	2,57 ± 1,35	3,21 ± 2,06	1,80 ± 1,66	0,5104
Catalase U/mgHb	0	521,1 ± 359,9	723,0 ± 254,1	79,91 ± 132,1	115,9 ± 96,67	0,0130*
	12	398,5 ± 604,7	147,3 ± 95,24	36,41 ± 11,95	64,97 ± 54,09	0,0842
	24	10,98 ± 5,36	51,93 ± 43,55	86,85 ± 52,48	62,67 ± 58,81	0,3181

Data expressed as mean and standard deviation. GI and GII - diet LSC 0.5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. a Statistical test - Kruskal-Wallis and Dunn test. *p <0.05 GII> GIII .

Table 4. Macroscopic analysis of the aortas according to the sudanofílica area.

Sudanophilic Area	**GI** (n=4)	**GII** (n=5)	**GIII** (n=4)	**GIV** (n=3)
0 - Absent	-	-	-	-
1 - Discreet	1	1	2	2
2 - Moderate	-	-	2	1
3 - Intense	3	4	-	-

GI and GII - diet LSC 0,5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. a Statistical test - Fischer exact test; p = 0.06823.

Table 5. Macroscopic evaluation of the% of plaques in the aorta.

% of plaque	**GI** (n=4)	**GII** (n=5)	**GIII** (n=4)	**GIV** (n=3)	**p(GIxGIIxGIIIxGIV)[a]**

% of aorta plaque	83,00 ± 24,62	84,64 ± 23,22	15,38 ± 5,95	24,11 ± 8,81	0,0091***
Aorta area	351,6 ± 328,0	261,1 ± 325,2	134,5 ± 62,4	124,1 ± 125,2	0,0036*
Plaque area	296,5 ± 101,5	223,4 ± 751,9	207,2 ± 80,7	304,7 ± 129,9	0,0072**

Data expressed in Mean and Standard Deviation. GI and GII - diet LSC 0.5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. Kruskal Wallis / Dunn test for comparison between groups * p <0.05 - GI> GIV; **p <0.05 - GI> GIII; *** p <0.05 - G II> III

Table 6. Calcification in the aorta.

Portion	**GI** (n=4)	**GII** (n=5)	**GIII** (n=4)	**GIV** (n=3)	**p(GIxGIIxGIIIxGIV)[a]**
Arch	4,59 ± 5,89	0,54 ± 0,34	2,55 ± 2,06	4,08 ± 0,0	0,4839
Thoracic	1,35 ± 2,34	0,08 ± 0,06	0,46 ± 0,15	0,73 ± 0,12	0,0513
Abdominal	0,22 ± 0,26	0,04 ± 0,03	1,29 ± 2,07	0,97 ± 0,26	0,0452*

Data expressed as Mean and Standard Deviation. GI and GII - diet LSC 0.5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. a Statistical test - Kruskal_Wallis test / Dunn test *p <0.05 - GII <GIV.

Table 7. Aortic Fibrosis Area.

Portion	**GI** (n=4)	**GII** (n=5)	**GIII** (n=4)	**GIV** (n=3)	**p(GIxGIIxGIIIxGIV)[a]**
Arch	8,29 ± 4,92	7,58 ± 3,79	11,24 ± 3,80	11,56 ± 5,37	0,4869
Thoracic	2,57 ± 1,68	2,63 ± 2,12	5,9 ± 5,48	2,87 ± 1,52	0,7379
Abdominal	1,77 ± 0,62	3,26 ± 1,23	3,21 ± 2,51	2,68 ± 0,58	0,3494

Data expressed as Mean and Standard Deviation GI and GII - diet LSC 0.5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. a Statistical test - Kruskal_Wallis test / Dunn test.

Table 8. Percentage of area immunostaining with the ICAM-1, MCP-1 and e-NOS antibodies in the aortic arch.

Antibody	**GI (n=4)**	**GII (n=5)**	**GIII (n=4)**	**GIV (n=3)**	**p(GIxGIIxGIIIxGIV)**
MCP-1	34,93 ± 24,95	24,67 ± 10,85	35,00 ± 15,87	6,07 ± 4,15	0,1072
ICAM1	5,81 ± 2,86	4,52 ± 4,15	0,55 ± 0,35	0,24 ± 0,22	0,0165*
e-NOS	37,07 ± 17,32	28,63 ± 8,70	15,57 ± 7,39	0,48 ± 0,45	0,0397**

GI and GII - diet LSC 0,5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. Statistical test - Kruskal-Wallis and Dunn's test - * p <0.05 GIV <GII; ** p <0.05 GIV <GI

With respect to the analysis of the thoracic and abdominal aorta, it was not observed significant difference between analyzed groups, however, the expression of MCP-1 in thoracic portion was smaller in GI and GII in relation to other groups. In addition, we observed that the expression of and us in the abdominal portion was smaller in the GI group when compared to other groups (Tables 9, 10; Figure 4).

Table 9. Percentage of area immunostaining with the ICAM-1, MCP-1 and e-NOS antibodies in the thoracic aorta.

Antibody	GI (n=4)	GII (n=5)	GIII (n=4)	GIV (n=3)	p(GIxGIIxGIIIxGIV)
MCP-1	19,91 ± 10,08	12,62 ± 10,52	37,55 ± 18,97	30,48 ± 0,73	0,1112
ICAM1	5,00 ± 4,88	4,08 ± 2,63	4,22 ± 5,31	3,63 ± 0,90	0,8999
e-NOS	15,97 ± 13,50	17,91 ± 2,39	13,86 ± 8,60	11,96 ± 4,76	0,575

GI and GII - diet LSC 0,5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. Statistical test -Kruskal-Wallis and Dunn's test.

Table 10. Percentage of area immunostaining with the ICAM-1, MCP-1 and e-NOS antibodies in the abdominal aorta.

Antibody	GI (n=4)	GII (n=5)	GIII (n=4)	GIV (n=3)	p(GIxGIIxGIIIxGIV))
MCP-1	32,71 ± 12,84	25,52 ± 23,06	27,82 ± 18,30	22,52 ± 25,56	0,8744
ICAM1	0,12 ± 0,13	4,29 ± 7,34	2,84 ± 3,16	3,79 ± 6,01	0,3198
e-NOS	8,73 ± 3,40	28,58 ± 22,40	13,67 ± 10,59	14,18 ± 6,45	0,2734

GI and GII - diet LSC 0.5 / LSC 0,1; GIII and GIV - LSC 0.5 / normal; GII and GIII - treated as vitamin D. Statistical test - Kruskal-Wallis and Dunn's test - p> 0.05.

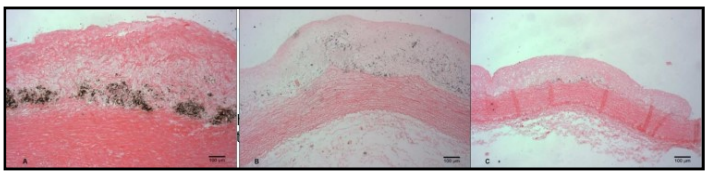

Figure 2. Photomicrograph of histological section of the aorta showing the regions with calcium. Coloring of Von Kossa. A - Aortic arch, B - Thoracic aorta, C Abdominal aorta.

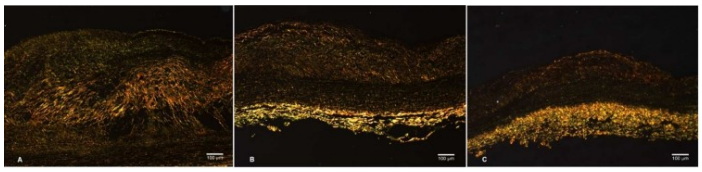

Figure 3. Photomicrograph of histological section of the aorta under polarized light, showing the regions with collagen deposition. Picro-sirius staining A - Aortic arch, B - Thoracic aorta, C Abdominal aorta.

With respect to aortic arch in expression of ICAM-1, MCP-1 and and-we can see that there was a significant difference between the groups and GI in relation to GIV immunolabeling of ICAM-1, however there is also a decrease of the expression of ICAM-1 in the Group GIII and GIV as well as MCP-1 in the Group GIV when compared to other groups and also showed a decrease in Group GIII, however, we observed significant difference only in GIV and GI (Table 8).

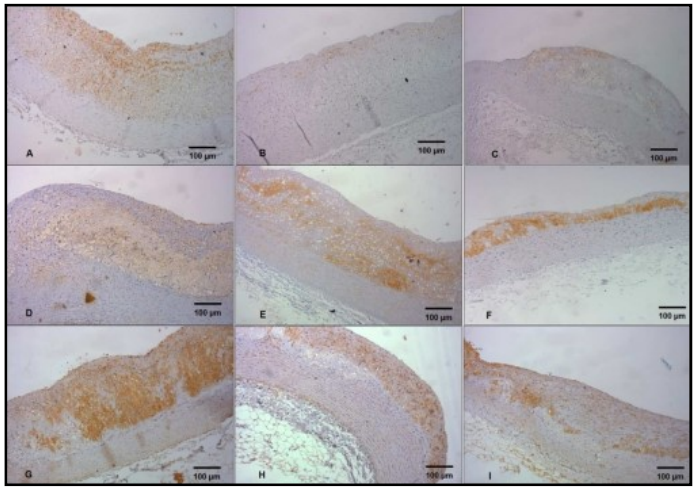

Figure 4. Immunostaining photomicrography of anti-ICAM-1 antibodies (A - aortic arch; B - thoracic aorta; C - abdominal aorta); anti-MCP-1 (D - aortic arch; E - thoracic aorta; F - abdominal aorta); anti-and-NOS (G - aortic arch, H - thoracic aorta, I - abdominal aorta).

Discussion

CVD specifically atherosclerosis has a high rate of morbidity and mortality in populations with age and that can have a better quality of life. Atherosclerosis is a multifactorial disease and widely linked to lifestyle and eating habits. In this sense, the search for food and/or dietary supplements that may prevent mitigate or even reverse the damage caused to the body by atherosclerosis and hypercholesterolemia, have led several

research groups investigating the possible effects of foods, vitamins or isolated substances. Many studies propose that vitamin D deficiency is not only related to the balance of calcium and phosphorus in bone metabolism, but also to several other diseases, such as cancer, autoimmune diseases, CVD, diabetes and other chronic diseases. The discovery of vitamin D receptors in other cells that not only the bony, aroused the interest in knowing how this hormone could act in other target organs[34,35,36,37]. Based on this question, researchers from different countries focused their goals in elucidating the possible effects that vitamin D might have with respect to prevention, stabilization and even improvement in the parameters involved in the pathogenesis of diseases related to lipid metabolism.

Starting from this assumption, our research is exploratory and we have proposed in this study to evaluate the possible effects of vitamin D3 in an experimental model of atherosclerosis induced by diet. We opted for the induction of atherogenesis by diet, as it is a well established protocol in both the literature and the research line of the group, moreover, closer to what occurs in humans. Initially randomized the animals in two large groups and do comparison with respect to the administration of vitamin D. along the test due to technical problems, we subdivided into four groups and, thereby, our comparison also covers the issue of normalization of diet after 12 weeks of consuming a high-fat diet. Unfortunately, during the experiment we lost a total of four animals, which has damaged our analysis because, due to the size of the sample, statistically we can't assert our findings, only inferred.

The animals kept the weight gain independent of the Group analyzed, we observe that the groups GIII and GIV, presented a slightly higher weight gain when compared to groups GI and GII, this difference is since the normalization of diet after the 12th week compared to those who remained the LSC diet until the end of the test. Food consumption, also there was no statistical difference between the groups, at the beginning of the experiment there is a somewhat higher consumption when compared to the final period. Two of the groups that kept the animals diet LSC throughout the Protocol stopped eating, showed diarrhea, jaundice and demonstrated apathy before any stimulus, for this reason, we need to remove the LSC diet until the feces if normalizes to the animals conclude the 24 weeks. Animals which had the standard diet had a slightly higher consumption, but still not significant. Our data consumption and body mass evolution in this model of induction of atherosclerosis by high-fat diet and hypercholesterolemic are according to the data published in the literature. Helfenstein et al. (2011)[38] also observed weight gain in the groups that received a diet atherogenic and default during the experimental protocol of 24 weeks.

Analyzing the lipid profile of our groups, we note that there is an improvement in parameters related to serum levels of Total cholesterol, triglycerides and VLDL-c, especially when the diet a paroxysmal tachycardia. The data for these parameters decreased considerably when we compare the values expressed in the 12th week in relation to the 24th week. It is important to observe that there was a significant difference in the mean values of Triglycerides, because levels above 150 mg/dL are considered as hypertriglyceridemia[39], in this way, our results indicate that the normalization of diet, associated or not to vitamin D managed to reduce plasma levels of this lipid fraction. It is postulated in the literature that high levels of Triglycerides are associated with hypercholesterolemia and atherosclerosis, as well as to reduce the levels of this lipid fraction decreases the risk of cardiovascular events[39]. In this sense, our data are in accordance with the results presented in literature, as in your study, Garavelo and collaborators (2017)[40] by means of an experimental model of atherosclerosis, noted that the animals showed an improvement in lipid profile, after treatment with the extract of a medicinal plant, associated with TRANS-Sialidase enzyme, this compound was developed especially for the analysis of effects on atherosclerosis associated to infection with Mycoplasma pneumoniae.

In another study, Malek and Shata (2014)[41], observed that after administration of a high dose of vitamin D (50000 IU per day) for a period of four weeks via intramuscular, levels of total cholesterol, triglycerides and LDL-c decreased, since the levels of HDL-c increased in compared to groups who have not received the injection of vitamin D. Because of the similarity of the experimental model used and comparing our data with those submitted by the cited studies, we can infer that if our sample was larger, it is likely that we could have obtained significant results in relation to serum parameters analyzed by the authors. However, the dose of vitamin D was very high compared to our share. We opted for the daily dose of 1000 IU per day, since most of the articles published in the literature using high doses of vitamin D and our proposal would be to analyze the results before a low dosage commonly spread among in the population and in any drugstore.

Another aspect as important as the lipid profile in the pathogenesis of atherosclerosis is related to the activity of antioxidant enzymes, as there is an increase in the levels of ROS during atherogenesis due to the inflammatory process. In this sense, the SOD and CAT enzyme activity is essential for maintaining homeostasis on the formation of ROS, because the imbalance between the production of ROS and protective enzymes activity, culminating in the oxidative stress. Oxidative stress is widely deleterious to the Agency and, in the process of the development of atherosclerosis, is involved in the inflammatory process and lipid peroxidation. Starting from the premise that vitamin D can act as an antioxidant, we analyzed our samples with activity levels of antioxidant enzymes SOD and CAT. Both showed a reduction in your activity in all groups when we compare the levels observed in the 12 weeks to the end of the study period, although not statistically significant this is important, especially if Mrs. Harmon--that the decrease of the SOD and CAT enzyme activity may be associated with the saturation of the enzymatic antioxidant defense mechanisms. On the other hand, maybe the reduction in levels of SOD and CAT might be linked to the fall in levels of ROS in atherogenesis, by the action of vitamin D, because it acts by reducing the levels of TNF-α by inhibiting the signaling pathway of NF-kB/p65[42]. To

elucidate this issue, further analysis could be made, such as measurement of levels of EROs and circulating levels of vitamin D.

Some authors relate the reduction in levels of SOD and CAT to a bad parameter, due to saturation of enzymatic defense mechanisms by ROS[14]. On the other hand, we could say that vitamin D acting as exogenous source, could have assisted in the reduction of ROS, thus saving the enzymatic defense system.

Farhangi and collaborators (2017)[43] also examined the enzymatic activity of SOD and CAT in an experimental model of rats fed a high-fat diet associated with the administration of 500 IU/Kg/day via gavage for 5 weeks. They observed that in groups where it was administered vitamin D, there was a decrease in enzymatic activity of SOD, and an increase in the CAT. The researchers justify this result to the compensatory adaptation of the organism to the oxidative stress caused by high-fat diet and point out that the SOD levels may have been lower in the group that received vitamin D due to vitamin uptake by fatty tissue, given your fat solubility, on the other hand, the CAT increase reflects the action of the enzyme in the Disproportionation of hydrogen peroxide, reducing their potential effects in the model organism analyzed[43]. Histopathological changes found in the tissues analyzed are in accordance with the results shared by literature, where we can observe that the hypercholesterolemic diet was able to induce the atherosclerotic lesions in animals. Analyzing the tissues, we found that the formation of atherosclerotic plaque in the aorta was lower in GIII and GIV when compared to other groups. These findings if corroborated when related to assessment of the percentage of boards with the macro and microscopic analysis associated with morphometric analysis of I/M relationship, because the groups were staged in GIV and GIII grade II and III, corresponding respectively to the content as discreet and moderate lipid, while the groups GI and GII were staged in Grades III and IV, with sudanophilic in your most intense stained. We could justify these findings inferring that the normalization of diet tends to improve the lipid profile which reflects directly on the histopathological findings, independent of the administration of vitamin D, however, as our sample is small, we have no way to say our results.

Satilmis and collaborators (2015)[44], found that there is an inverse correlation between vitamin D levels and the characteristics of atherosclerotic plaque, and low levels are linked to the severity of atherosclerosis in young adults[5]. Wang and Zhang (2017)[5] found a positive correlation between serum levels of vitamin D and the I/M ratio in patients with type II diabetes. The relationship between the I/M ratio is associated with Subclinical Atherosclerosis risks.

The deposition of calcium and the area of aortic tissue fibrosis show that the groups I and II had a better result when compared to the GIII and GIV. The literature makes it clear that one of the factors that may contribute to the deposition of calcium during atherosclerosis is related to injury to the endothelium by oxidative stress, mainly by superoxide anion, with reduced enzyme activity of SOD. Associated with this picture, the hydrogen peroxide promotes a phenotypic change of smooth muscle cells to cells with osteogenic potential, which favors even more calcium deposition in the aorta.

Other studies, advanced glycation end products related to the calcification of atherosclerotic plaque resulting from hyperglycemia[45]. Our analyses do not showed hyperglycemia, nor the development of diabetes, we can suggest that the deposition of calcium in the aortas of rabbits can be as a result of injuries caused by inflammatory and oxidative process that can lead to change in smooth muscle cell phenotype.

Immunohistochemistry analysis, we observed significant difference in percentage of immunostaining among groups Island GII and GIV, we can infer that the normalization of diet decreases the expression of insulin, being considered a positive result when compared to other groups. Yet in this regard, ICAM-1 was also significant between the groups GI and GIV evidencing that there has been a decrease in the expression of inflammatory molecule. With respect to the MCP-1 and markers and us we observe that there is a reduction in the percentage of immunostaining area of MCP-1 between the groups GII, GIII and GIV, which means there is a decrease in the inflammatory process and showed lower expression in GIV when compared to other groups, which leads us to believe there is a modulation of inflammatory parameters that may have been improved as a result of normalization of diet.

We analyze the profile of inflammatory markers in aortic tissue. The data show that there has been a significant result on immunolabeling of ICAM-1 in aortic arch among groups GIV and GII, as well as the expression of and between GIV and GI, MCP-1 also showed a decrease when compared with the other GIV group groups. The data indicate that the diet normalization not only improved the lipid profile, but also inflammatory. With respect to the thoracic portion, MCP-1 showed a decrease in GI and GII, accordingly we could suggest that vitamin D may have exercised some effect on this group, both ICAM-1, as e-introduced similar data in both groups. In the abdominal portion MCP-1 presented lower immunoexpression in the GIV Group in relation to the other groups, since ICAM-1 and e-NOS presented lower results in the GI Group.

Our data are consistent with those presented by Malek and Shata (2014)[41], the authors also observed a reduction in the levels of ICAM-1 in rabbits fed a high-fat diet and a high dose of vitamin D they associated the reduction of inflammatory molecules to Vitamin action. In another study, Bozic et al. (2015)[9] analyzed the relationship between vitamin D receptors and inflammatory markers that participate in the development of aterogênese. There is a relationship between the loss of the vitamin D receptor and Endothelial Activation with the increase of inflammatory molecules, both in vitro as in vivo analysis[9].

In their study, Li et al. (2015)[42] analyzed the levels of e-NOS by Western Blot and verified that e-NOS levels were lower in the groups that did not receive astaxanthin when compared to the astaxanthin groups, showing that the decrease in e and-NOS is related to the progression of atherogenesis.

Based on the above and due to the size of our sample, we can only suggest based on the publications reviewed and the trend of our results that vitamin D associated with the normalization of diet could improve the biochemical parameters related to lipid profile, still, about other parameters such as the activity of oxidative enzymes and the behavior of the molecules that participate in the inflammatory process, in this study, we cannot conclude with accuracy. That way, other analysis such as dosage of vitamin D serum levels and analysis of EROs would need to be made to elucidate the mechanisms of action of vitamin D on experimental atherosclerosis model.

Conclusion

In this experimental model of diet-induced atherosclerosis, antiinflammatory and antioxidant effects of vitamin D evaluated in serum and aortic tissues were not observed. The number of animals was one of the limiting factors of our study, as well as the lack of financial resources for other analyzes.

Conflict of interest

No conflict of interest.

Acknowledgement

None

References

1. World Health Organization. Mortality and global health estimates 2017 [Available from: http://www.who.int/gho/mortality_burden_disease/en/
2. Mansur AP, Favarato D. Trends in mortality rate from cardiovascular disease in Brazil, 1980-2012. Arq Bras Cardiol. 2016;107(1):20-25.
3. Marzilli M, Merz CN, Boden WE, Bonow RO, Capozza PG, Chilian WM, DeMaria AN, Guarini G, Huqi A, Morrone D, Patel MR, Weintraub WS. Obstructive coronary atherosclerosis and ischemic heart disease: an elusive link! J Am Coll Cardiol. 2012;60(11):951-956. doi: 10.1016/j.jacc.2012.02.082
4. van Ballegooijen AJ, Robinson-Cohen C, Katz R, Criqui M, Budoff M, Li D, Siscovick D, Hoofnagle A, Shea SJ, Burke G, de Boer IH, Kestenbaum B. Vitamin D metabolites and bone mineral density: The multi-ethnic study of atherosclerosis. Bone. 2015;78:186-193. doi: 10.1016/j.bone.2015.05.008
5. Wang Y, Zhang H. Serum 25-Hydroxyvitamin D3 levels are associated with carotid intima-media thickness and carotid atherosclerotic plaque in type 2 diabetic patients. J Diabetes Res. 2017;2017:3510275. doi: 10.1155/2017/3510275
6. Chai M, Zhang HT, Zhou YJ, Ji QW, Yang Q, Liu YY, Zhao YX, Shi DM, Liu W, Yang LX, Zhang LL, Liang J. Elevated IL-37 levels in the plasma of patients with severe coronary artery calcification. J Geriatr Cardiol. 2017;14(5):285-291. doi: 10.11909/j.issn.1671-5411.2017.05.013
7. Zakiev ER, Nikiforov NG, Orekhov AN. Cell-based models for Development of antiatherosclerotic therapies. Biomed Res Int. 2017;2017:5198723. doi: 10.1155/2017/5198723
8. Turko IV, Marcondes S, Murad F. Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA:3-oxoacid CoA-transferase. Am J Physiol Heart Circ Physiol. 2001;281(6):H2289-94. doi: 10.1152/ajpheart.2001.281.6.H2289
9. Bozic M, Álvarez Á, de Pablo C, Sanchez-Niño MD, Ortiz A, Dolcet X, Encinas M, Fernandez E, Valdivielso JM. Impaired vitamin D signaling in endothelial cell leads to an enhanced leukocyte-endothelium interplay: implications for atherosclerosis development. PLoS One. 2015;10(8):e0136863. doi: 10.1371/journal.pone.0136863
10. Al-Nimer MS, Hussein II. Increased mean carotid intima media thickness in type 2 diabetes mellitus patients with non-blood pressure component metabolic syndrome: A preliminary report. Int J Diabetes Dev Ctries. 2009;29(1):19-22. doi: 10.4103/0973-3930.50710
11. Maritim AC, Sanders RA, Watkins JB 3rd. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol. 2003;17(1):24-38. doi: 10.1002/jbt.10058
12. Halliwell B, Gutteridge JM. Biologically relevant metal ion-dependent hydroxyl radical generation. An update. FEBS Lett. 1992;307(1):108-112. doi: 10.1016/0014-5793(92)80911-y
13. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr Rev. 2002;23(5):599-622. doi: 10.1210/er.2001-0039
14. Lubrano V, Balzan S. Enzymatic antioxidant system in vascular inflammation and coronary artery disease. World J Exp Med. 2015;5(4):218-224. doi: 10.5493/wjem.v5.i4.218
15. Gupta S, Sodhi S, Mahajan V. Correlation of antioxidants with lipid peroxidation and lipid profile in patients suffering from coronary artery disease. Expert Opin Ther Targets. 2009;13(8):889-894. doi: 10.1517/14728220903099668
16. Eftekhari MH, Aliasghari F, Beigi MA, Hasanzadeh J. The effect of conjugated linoleic acids and omega-3 fatty acids supplementation on lipid profile in atherosclerosis. Adv Biomed Res. 2014;3:15. doi: 10.4103/2277-9175.124644
17. Oosterwerff MM, Eekhoff EM, Heymans MW, Lips P, van Schoor NM. Serum 25-hydroxyvitamin D levels and the metabolic syndrome in older persons: a population-based study. Clin Endocrinol (Oxf). 2011;75(5):608-613. doi: 10.1111/j.1365-2265.2011.04110.x
18. Cozzolino M, Stucchi A, Rizzo MA, Soldati L, Cusi D, Ciceri P, Brenna I, Elli F, Gallieni M. Reprint of: Vitamin D

receptor activation and prevention of arterial ageing. Nutr Metab Cardiovasc Dis. 2013;23 Suppl 1:S31-6. doi: 10.1016/j.numecd.2012.11.001

19. Hosseinpanah F, Yarjanli M, Sheikholeslami F, Heibatollahi M, Eskandary PS, Azizi F. Associations between vitamin D and cardiovascular outcomes; Tehran Lipid and Glucose Study. Atherosclerosis. 2011;218(1):238-242. doi: 10.1016/j.atherosclerosis.2011.05.016

20. Fiscella K, Franks P. Vitamin D, race, and cardiovascular mortality: findings from a national US sample. Ann Fam Med. 2010;8(1):11-18. doi: 10.1370/afm.1035

21. Artaza JN, Mehrotra R, Norris KC. Vitamin D and the cardiovascular system. Clin J Am Soc Nephrol. 2009;4(9):1515-1522. doi: 10.2215/CJN.02260409

22. Maeda SS, Borba VZC, Camargo MBR, Silva DMW, Borges JLC, Bandeira F, Lazaretti-Castro M.. Recommendations of the Brazilian Society of Endocrinology and Metabology (SBEM) for the diagnosis and treatment of hypovitaminosis D. Arq Bras Endocrinol Metab. 2014;58(5):411-433.

23. Wang H, Chen W, Li D, Yin X, Zhang X, Olsen N, Zheng SG. Vitamin D and chronic diseases. Aging Dis. 2017;8(3):346-353. doi: 10.14336/AD.2016.1021

24. Pérez-Hernández N, Aptilon-Duque G, Nostroza-Hernández MC, Vargas-Alarcón G, Rodríguez-Pérez JM, Blachman-Braun R. Vitamin D and its effects on cardiovascular diseases: a comprehensive review. Korean J Intern Med. 2016;31(6):1018-1029. doi: 10.3904/kjim.2015.224

25. Castro LCG. O sistema endocrinológico vitamina D. Arq Bras Endocrinol Metab. 2011;55(8):566-575. http://dx.doi.org/10.1590/S0004-27302011000800010

26. Waqar AB, Koike T, Yu Y, Inoue T, Aoki T, Liu E, Fan J. High-fat diet without excess calories induces metabolic disorders and enhances atherosclerosis in rabbits. Atherosclerosis. 2010;213(1):148-155. doi: 10.1016/j.atherosclerosis.2010.07.051

27. Kolodgie FD, Katocs AS Jr, Largis EE, Wrenn SM, Cornhill JF, Herderick EE, Lee SJ, Virmani R. Hypercholesterolemia in the rabbit induced by feeding graded amounts of low-level cholesterol. Methodological considerations regarding individual variability in response to dietary cholesterol and development of lesion type. Arterioscler Thromb Vasc Biol. 1996;16(12):1454-1464. doi: 10.1161/01.atv.16.12.1454

28. Moghadasian MH. Experimental atherosclerosis: a historical overview. Life Sci. 2002;70(8):855-865. doi: 10.1016/s0024-3205(01)01479-5

29. Dornas WC, Oliveira TT, Augusto LE, Nagem TJ. Experimental atherosclerosis in rabbits. Arq Bras Cardiol. 2010;95(2):272-278. doi: 10.1590/s0066-782x2010001200020

30. Holvoet P, Collen D. beta-VLDL hypercholesterolemia relative to LDL hypercholesterolemia is associated with higher levels of oxidized lipoproteins and a more rapid progression of coronary atherosclerosis in rabbits. Arterioscler Thromb Vasc Biol. 1997;17(11):2376-2382. doi: 10.1161/01.atv.17.11.2376.

31. Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Circulation. 1995;92(5):1355-1374. doi: 10.1161/01.cir.92.5.1355

32. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem. 1969;244(22):6049-6055. https://www.jbc.org/article/S0021-9258(18)63504-5/pdf

33. Adamo AM, Llesuy SF, Pasquini JM, Boveris A. Brain chemiluminescence and oxidative stress in hyperthyroid rats. Biochem J. 1989;263(1):273-277. doi: 10.1042/bj2630273

34. He XJ, Ding Y, Xiang W, Dang XQ. Roles of 1,25(OH)2D3 and vitamin D receptor in the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus by regulating the activation of CD4+ T cells and the pkcδ/erk signaling pathway. Cell Physiol Biochem. 2016;40(3-4):743-756. doi: 10.1159/000453135

35. Fondjo LA, Owiredu WKBA, Sakyi SA, Laing EF, Adotey-Kwofie MA, Antoh EO, Detoh E. Vitamin D status and its association with insulin resistance among type 2 diabetics: A case -control study in Ghana. PLoS One. 2017;12(4):e0175388. doi: 10.1371/journal.pone.0175388

36. Wang WH, Chen LW, Lee CC, Sun CY, Shyu YC, Hsu HR, Chien RN, Wu IW. Association between parathyroid hormone, 25 (OH) vitamin D, and chronic kidney disease: a population-based study. Biomed Res Int. 2017;2017:7435657. doi: 10.1155/2017/7435657

37. Yang Y, Zhang X, Bao M, Liu L, Xian Y, Wu J, Li P. Effect of serum 25-hydroxyvitamin D3 on insulin resistance and β-cell function in newly diagnosed type 2 diabetes patients. J Diabetes Investig. 2016;7(2):226-232. doi: 10.1111/jdi.12381

38. Helfenstein T, Fonseca FA, Ihara SS, Bottós JM, Moreira FT, Pott H Jr, Farah ME, Martins MC, Izar MC. Impaired glucose tolerance plus hyperlipidaemia induced by diet promotes retina microaneurysms in New Zealand rabbits. Int J Exp Pathol. 2011;92(1):40-49. doi: 10.1111/j.1365-2613.2010.00753.x

39. Carroll M, Kit B, Lacher D. Trends in elevated triglyceride in adults: United States, 2001-2012. NCHS Data Brief.

2015;(198):198.
https://www.cdc.gov/nchs/data/databriefs/db198.pdf

40. Garavelo SM, Higuchi ML, Pereira JJ, Reis MM, Kawakami JT, Ikegami RN, Palomino SA, Wadt NS, Agouni A. Comparison of the protective effects of individual components of particulated trans-sialidase (PTCTS), PTC and TS, against high cholesterol diet-induced atherosclerosis in rabbits. Biomed Res Int. 2017;2017:7212985. doi: 10.1155/2017/7212985

41. Malek HA, Shata A. Effect of a high dose of vitamin D on a rabbit model of atherosclerosis. Int J Immunopathol Pharmacol. 2014;27(2):195-201. doi: 10.1177/039463201402700206

42. Li P, Pan GP, Jia M, Wang QQ, Guo ZG, Zhao FR, Lei GL, Wan GR, Wan GM. Effect of Xin Mai Jia on atherosclerosis in rats. Genet Mol Res. 2015;14(2):6018-6027. doi: 10.4238/2015.June.1.19

43. Farhangi MA, Nameni G, Hajiluian G, Mesgari-Abbasi M. Cardiac tissue oxidative stress and inflammation after vitamin D administrations in high fat- diet induced obese rats. BMC Cardiovasc Disord. 2017;17(1):161. doi: 10.1186/s12872-017-0597-z

44. Satilmis S, Celik O, Biyik I, Ozturk D, Celik K, Akın F, Ayca B, Yalcin B, Dagdelen S. Association between serum vitamin D levels and subclinical coronary atherosclerosis and plaque burden/composition in young adult population. Bosn J Basic Med Sci. 2015;15(1):67-72. doi: 10.17305/bjbms.2015.238

45. Wei Q, Ren X, Jiang Y, Jin H, Liu N, Li J. Advanced glycation end products accelerate rat vascular calcification through RAGE/oxidative stress. BMC Cardiovasc Disord. 2013;13:13. doi: 10.1186/1471-2261-13-13