Vitamin D Induces Increased Systolic Arterial Pressure via Vascular Reactivity and Mechanical Properties

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Abstract

**Background/Aims:** The aim of this study was to evaluate whether supplementation of high doses of cholecalciferol for two months in normotensive rats results in increased systolic arterial pressure and which are the mechanisms involved. Specifically, this study assesses the potential effect on cardiac output as well as the changes in aortic structure and functional properties.

**Methods:** Male Wistar rats were divided into three groups: 1) Control group (C, n = 20), with no supplementation of vitamin D; 2) VD3 (n = 19), supplemented with 3,000 IU vitamin D/kg of chow; 3) VD10 (n = 21), supplemented with 10,000 IU vitamin D/kg of chow. After two months, echocardiographic analyses, measurements of systolic arterial pressure (SAP), vascular reactivity, reactive oxygen species (ROS) generation, mechanical properties, histological analysis and metalloproteinase-2 and -9 activity were performed.

**Results:** SAP was higher in VD3 and VD10 than in C rats (p = 0.001). Echocardiographic variables were not different among groups. Responses to phenylephrine in endothelium-denuded aortas was higher in VD3 compared to the C group (p = 0.041). Vascular relaxation induced by acetylcholine (p = 0.023) and sodium nitroprusside (p = 0.005) was impaired in both supplemented groups compared to the C group and apocynin treatment reversed impaired vasodilation. Collagen volume fraction (<0.001) and MMP-2 activity (p = 0.025) was higher in VD10 group compared to the VD3 group. Elastin volume fraction was lower in VD10 than in C and yield point was lower in VD3 than in C.

**Conclusion:** Our findings support the view that vitamin D supplementation increases arterial pressure in normotensive rats and this is associated with structural and functional vascular changes, modulated by NADPH oxidase, nitric oxide, and extracellular matrix components.

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Introduction

Vitamin D (VD) is a fat-soluble compound primarily obtained through cutaneous synthesis. The remainder of VD may be obtained from supplements or foods, but few foods contain substantial amounts of VD [1,2].

The prevalence of VD deficiency has increased in recent years [3], becoming a public health problem worldwide [4]. It is estimated that one billion people worldwide are either VD insufficient or deficient [3]. Furthermore, VD deficiency is associated with an increased risk of developing several chronic diseases [2,5,6,7,8,9,10,11]. Therefore, researchers have recommended increased sun exposure, food fortification and VD supplementation, both for people at higher risk for hypovitaminosis D and for the general population [12,13,14]. Medium and long term effects of VD supplementation with doses above 4,000 IU/day are not well known, and risks may not be disregarded, although toxic effects are rare [15]. VD dose of 4,000 IU/day was recently recommended by Institute of Medicine as Upper Level Intake [16].

The classic function of VD is to regulate calcium and phosphorous homeostasis, but VD also modulates the function of a variety of non-classical target tissues, including vascular smooth muscle cells (VSMC) and endothelial cells [17,18,19]. Several mechanisms have been proposed on how VD could be involved in blood pressure regulation and the pathophysiology of arterial hypertension. VD effects on the renin angiotensin aldosterone system (RAAS), by down-regulates renin expression, have been
extensively investigated by experimental studies [20,21]. Other mechanisms linking VD and blood pressure may be related to direct VD effects on the vasculature [22,23,24,25].

Several randomized controlled trials (RCTs) evaluating the effects of VD supplementation on blood pressure have been conducted with inconsistent results [26,27,28,29,30,31]. These results along with some other RCTs showing no significant blood pressure effect of VD in largely normotensive individuals [27,32,33,34], suggest that if antihypertensive effects of VD are actually present, these may only be observed in groups with both low VD levels and high blood pressure [35].

On the other hand, in experimental studies Bukoski & Xue (1993) [36] and Haffner et al. (2005) [37] showed that administering 1,25-dihydroxyvitamin D increases systolic blood pressure in normotensive rats. In agreement with this result, our group has shown that normotensive rats supplemented for two months with cholecalciferol also presented with higher systolic blood pressure [38].

Systemic arterial blood pressure is determined by the cardiac output and systemic vascular resistance [39,40]. Vascular resistance is set predominantly by the vascular tone in the arterial tree but is also influenced by alterations in the vascular structural, functional and mechanical properties. Structural and functional abnormalities in the vasculature may be due to endothelial dysfunction, increased vascular oxidative stress, vascular remodeling, and decreased compliance. These factors directly impact vascular resistance and may antedate hypertension and contribute to its pathogenesis. Therefore, endothelial dysfunction, increased oxidative stress, vascular remodeling and decreased compliance directly impact vascular resistance [40,41]. Experimental studies with rats and cultured cells have shown that VD supplementation is associated with some of these vascular changes [22,23,24,25]. However, the mechanisms through which VD increases systolic arterial pressure are unclear.

Therefore, we tested the hypothesis that increased systolic arterial pressure in normotensive rats after VD supplementation results from both an increase in cardiac output and vascular resistance.

The aim of this study was to evaluate whether supplementing high doses of cholecalciferol for two months in normotensive rats increases systolic arterial pressure and which are the mechanisms involved. Specifically, this study assesses the potential effect on cardiac output as well as the changes in aortic structure and functional properties.

**Materials and Methods**

**Experimental protocol**

All experiments and procedures were performed in accordance with the National Institute of Health’s Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics Committee for Animal Experimentation of the Botucatu Medical School, UNESP, São Paulo, Brazil (2008/694). Male Wistar rats weighting 250 g were randomly allocated into three groups and fed a cereal-based chow diet for two months: 1) control group (C, n = 20), with no supplementation of VD (Cereal-based diet - Nuvilab CR1, with the approximate composition (kg mixture): protein, 220 g; fat, 40 g; mineral, 100 g; fiber, 80 g and VD, 1,800 IU); 2) VD3 (n = 19), supplemented with 3,000 IU VD/kg of chow. All animal groups received 10 ml of corn oil per kg of chow. Supplementation with VD was made by adding cholecalciferol (Sigma-Aldrich, MO, USA) diluted in the corn oil. The National Research Council recommended to rats the amount of 1,000 IU of VD per kg of chow for rats [42]. However, they do not have defined an upper intake level. Therefore, we use the relation of ten times the recommended daily dose to have our high dose. Shepard & Deluca (1980) showed that rats supplemented with doses above 1,000 IU of VD/day (~ 30,000 IU/kg of chow) presented toxicity signs such as irritability, diarrhea, loss of appetite, decrease in weight gain, the kidneys became mottled and in their kidneys to take on a grayish-white color indicative of calcification [43]. The doses used in our study were 4.8 and 11.8 times higher than recommended dose for rats and did not reach the 1,000 IU/day considered toxic by Shepard & Deluca (1980) [43]. Therefore, the doses used in the present study were considered non toxic.

After two months of VD supplementation, the rats were submitted to measurements of systolic arterial pressure (SAP) and echocardiographic analyses. Thus, the animals were euthanized, and the thoracic aortas from each animal was carefully removed, and the segments were used to analyze the vascular reactivity, assessment of vascular reactive species, mechanical proprieties, histological analysis and metalloproteinase-2 and -9 activity.

**Systolic arterial pressure**

The systolic arterial pressure of the tail was measured one week before euthanasia with a tail plethysmograph. The animals were warmed in a wooden box at 40°C with heat generated by two incandescent lamps for four minutes to cause vasodilation artery tail and were then transferred to an iron cylindrical support that was specially designed to allow total exposure of the animal’s tail. A sensor (KSM-microphone) was placed in the proximal region of the tail, coupled to an electro-phygmonomanometer, Narco Bio-System, model 709-0610 (International Biomedical Inc, TX, USA) [44]. The electro-phygmonomanometer was attached to a computer where the systolic arterial pressure was measured with the software Biopac Student Lab PRO (Biopac Systems Inc., CA, USA).

**Echocardiographic study**

After 2 months, all animals were weighed and evaluated with a transthoracic echocardiographic exam [45]. The exams were performed using a commercially available echocardiographic machine (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipped with 5–12 MHz phased array transducer. All measurements were obtained by the same observer according to the leading-edge method recommended by the American Society of Echocardiography/European Association of Echocardiography [46]. The data represent the mean of measurements from at least five consecutive cardiac cycles. The rats were lightly anaesthetized with an intramuscular injection of a solution composed of ketamine (50 mg/kg) plus xylazine (1 mg/kg). The rat chests were shaved, and the rats were placed in a left lateral position. Targeted 2-D M-mode echocardiograms were obtained from short-axis views of the left ventricle (LV) at or just below the tip of the mitral-valve leaflets and at the level of the aortic valve and left atrium. M-mode images of the LV and left atrium were recorded at a sweep speed of 100 mm/s. The LV end-diastolic dimension (LVEDD) was measured at maximal diastolic dimension. The left atrium was measured at its maximal diameter. The LV systolic function was assessed by calculating the ejection fraction [(LVEDD3 – LVESD3)/LVEDD3]. The transmirtal diastolic flow (E and A) velocities were obtained from the apical four-chamber view. The E/A ratio was used as an index of the LV diastolic function.
Vascular reactivity

The thoracic aorta was isolated and cleaned of connective tissue and fat. Aortic rings, 4 mm in length, were cut and mounted for isometric tension recording. The rings were placed in bath chambers (5 ml) for isolated organs (Mulvany Myograph) [47] containing modified Krebs salt solution of the following composition (mM): NaCl 130, CaCl2 1.6, MgSO4 1.2, KH2PO4 1.2, KCl 4.7, NaHCO3 14.9, glucose 5.5, which was maintained at 37°C, pH 7.4, and bubbled with 95% O2 and 5% CO2 [48]. The responses were recorded on a computer system using the Chart V4.04, PowerLab ADInstruments (2000) program. The aortic rings were submitted to a tension of 30 mN during a 45-min equilibration period. In some aortic rings, the endothelium was gently removed with a needle. After equilibration, rings were pharmacologically tested for endothelial integrity with 60 mmol/L phenylephrine (Phe) (10^-10 to 10^-5 M) which was incubated for 30 min in the presence of N2. After the relaxation induced by Ach and NPS, the aortas were immediately fixed with grasping screws. The stretching speed was 30 mm/minute and a 50-N load was used. Failure load, yield point (by Johnson’s method), and stiffness were obtained. Failure load may be defined as the highest load tolerated by materials until rupture. Yield point is the maximum tension value below which materials comply with Hooke’s law (in which the tension-deformation function is linear). Beyond the yield point, some degree of lesion may be found and plastic deformation materials may already be present, making the return to initial length impossible even if the loading stops. Stiffness is the linear and constant numeric relation between load and elongation calculated at the yield point [52].

Mechanical properties analysis of the aorta

Aortic mechanical properties were studied in rat thoracic aortas. Before mechanical testing, 2-mm aorta fragments were promptly immersed in saline solution containing 0.25 mg/mL of papaverine to relax the muscle bundles of the arteries and to standardize the state of muscle tension in all aortic samples. The mechanical analysis was performed using a EMIC DL 10.000 Universal Machine of Mechanical Assays (Equipments and Testing Systems, Ltd., PR, Brazil). The aortas were immediately fixed with grasping clamps using smooth non-cutting metallic bars fastened with two screws. The stretching speed was 30 mm/minute and a 50-N load cell was used. Failure load, yield point (by Johnson’s method), and stiffness were obtained. Failure load may be defined as the highest load tolerated by materials until rupture. Yield point is the maximum tension value below which materials comply with Hooke’s law (in which the tension-deformation function is linear). Beyond the yield point, some degree of lesion may be found and plastic deformation materials may already be present, making the return to initial length impossible even if the loading stops. Stiffness is the linear and constant numeric relation between load and elongation calculated at the yield point [52].

Metalloproteinase-2 and -9 activities by gelatin zymography

The metalloproteinase (MMP)-2 and -9 activities were determined, as reported previously [53]. Briefly, the aortic samples were homogenized in buffer containing 50 mM Tris, pH 7.4: 0.2 M NaCl, 0.1% Triton X and 10 mM CaCl2. The tissue extracts were subjected to electrophoresis on 8% SDS-polyacrylamide containing 1% gelatin. Electrophoresis was performed in a Bio-Rad apparatus at 100 V for 2 h at 4°C. After electrophoresis, the gel was incubated for 1 h at room temperature in 2.5% SDS-polyacrylamide containing 1% gelatin. Electrophoresis was performed in a Bio-Rad apparatus at 100 V for 2 h at 4°C. After electrophoresis, the gel was incubated for 1 h at room temperature in 2.5% SDS-polyacrylamide containing 1% gelatin. Electrophoresis was performed in a Bio-Rad apparatus at 100 V for 2 h at 4°C. After electrophoresis, the gel was incubated for 1 h at room temperature in 2.5% SDS-polyacrylamide containing 1% gelatin. Electrophoresis was performed in a Bio-Rad apparatus at 100 V for 2 h at 4°C. After electrophoresis, the gel was incubated for 1 h at room temperature in 2.5% SDS-polyacrylamide containing 1% gelatin. Electrophoresis was performed in a Bio-Rad apparatus at 100 V for 2 h at 4°C. After electrophoresis, the gel was incubated for 1 h at room temperature in 2.5% SDS-polyacrylamide containing 1% gelatin. Electrophoresis was performed in a Bio-Rad apparatus at 100 V for 2 h at 4°C. After electrophoresis, the gel was incubated for 1 h at room temperature in 2.5% SDS-polyacrylamide containing 1% gelatin.
Results

The daily intake of VD, plasma 25(OH)D3 and serum calcium and phosphorus are listed in Table 1. The daily intake of VD and serum calcium and phosphorus were higher in supplemented groups compared to control. Plasma 25(OH)D3 was higher in VD10 than control.

The data on systolic blood pressure and echocardiographic variables are listed in Table 2. Systolic arterial pressure was higher in both the supplemented groups compared to the control. The echocardiographic variables (i.e., heart rate, cardiac output, systolic and diastolic function and morphological variables) were not different when comparisons were performed among the three groups.

The blood pressure was not associated with serum calcium (r = 0.05 e p = 0.77). Table 3 shows pharmacological parameters obtained from the cumulative concentration-response curves to Phe performed in endothelium-intact and endothelium-denuded aortas. Supplementation with 3,000 IU of VD significantly showed higher aortic Phe pD2 values in the endothelium-denuded aortas compared to the control group. No changes in the Phe maximal response were observed. However, changes in Phe responses were not observed in the endothelium-intact aortas.

These results are also listed in Table 3. The Ach pD2 values were not different among the groups. However, the maximal relaxation induced by Ach was significantly lower in the VD10 group than in the control group. Apocynin increased the Ach pD2 relaxation induced by Ach was significantly lower in the VD10 group compared to the control groups. The NADPH inhibitor apocynin restored the NPS relaxation.

Table 4 summarizes the results from histological analysis. The morphological data were not different when comparisons were performed among the three groups. However, the elastin volume fraction was lower, and the collagen volume fraction and the collagen/elastin ratio were higher in the VD10 group compared to the others groups. Moreover, the media of these animals (VD10) presented fragmentation of elastic fibers, which were observed in the arterial media of the samples from the VD10 group (Figure 1).

Vascular mechanical properties are listed in Table 5. Supplementation with 3,000 IU of VD significantly had a lower aorta yield point compared to the control. No differences were observed among the groups for the other mechanical variables, failure load and stiffness.

The data describing the MMP-2 and MMP-9 activity are listed in Table 5. The ratio for active/inactive MMP-2 was higher in the VD10 group compared to the VD3 group. The ratios for the active/inactive forms of MMP-9 were not different when comparisons were performed among the three groups.

Discussion

This study showed that VD supplementation for two months in normotensive rats is associated with higher arterial systolic pressure in these animals. In addition, there was higher aortic contractility, impairment of aortic relaxation, higher production of ROS, changes in collagen and elastin content and impairment of mechanical properties in the supplemented groups. These changes may be part of some mechanisms involved in the higher blood pressures that were found in the animals supplemented with VD.

In our study the animals supplemented with VD presented higher plasma 25(OH)D3. These values were consistent with data shown in studies which supplemented similar doses of VD [43,57]. The rats receiving both VD doses showed a slight increase in serum calcium but were still normocalcemic [43,57,58,59]. In addition, levels of 25(OH)D3 and calcium are below the values considered capable of generating toxicity signs by VD, as shown by Shepard and Deluca [43].

Consistent with previous reports [36,37,38], our data showed that VD supplementation in normotensive rats is associated with higher blood pressure. The elevation on the blood pressure was not associated with cardiac output nor with serum calcium in this model.

Over the past 2 decades, it has become apparent that VD is a modulator of vascular function [17]. Some VD actions are related to the increased vascular contractility [36,60,61,62]. In our study, we found that VD supplementation led to an increased contractile response to Phe in aortas with denuded endothelium. These data

| Variable               | C            | VD3          | VD10         | p       |
|------------------------|--------------|--------------|--------------|---------|
| Food ingestion (g/day)  | 25.5±1.5 (20)| 25.9±2.2 (19)| 25.2±2.2 (21)| 0.681   |
| Vitamin D ingestion    |              |              |              |         |
| (IU/day)               | 45.3 (44.2–48.2) (20) | 126.3 (116.7–128.5) (19) | 309.2 (288.6–317.6) * (21) | <0.001  |
| 25 (OH) D3 (ng/ml)     | 15.0 (13.2–20.7) (5) | 25.5 (19.0–40.5) (5) | 37.0 (34.1–40.0) * (5) | 0.016   |
| Ca (mg/dl)             | 8.24±0.366 (20) | 9.32±0.32 (19) | 9.44±0.15 (21) | 0.416   |
| P (mg/dl)              | 5.90 (5.65–6.10)* (20) | 6.80 (6.35–8.00)* (19) | 7.60 (6.95–8.78)* (21) | <0.001  |

Data are expressed as mean ± standard deviation of mean or median with 25 and 75 percentiles. Numbers in parentheses indicate the numbers of animals included in each experimental group. C: control group (no supplementation with vitamin D); VD3: supplemented with 3,000 IU VD/kg of chow; VD10: supplemented with 10,000 IU VD/kg of chow; 25 (OH) D3: plasma 25-hydroxycholecalciferol; Ca: serum calcium; P: serum phosphorus. * p<0.05 versus control group; # p<0.05 versus VD3 group.

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25 (OH) D3 (ng/ml)
are in accordance with other studies that demonstrated that 1,25-
dihydroxyvitamin D increased the contractile force-generating
capacity of the aorta and mesenteric arteries in both normotensive
and hypertensive rats [36,60,62]. When the experiment was
performed with endothelium, the contractile response returned to
normal. We hypothesize that the endothelium possesses relaxation
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capacity of the aorta and mesenteric arteries in both normotensive
and hypertensive rats [36,60,62]. When the experiment was
performed with endothelium, the contractile response returned to
normal. We hypothesize that the endothelium possesses relaxation
factors that controls the VD vasoconstriction effect. Therefore, this
vasoconstriction effect alone does not explain the blood pressure
elevation.

In addition, the animals supplemented with VD had impaired
relaxation to both Ach and SNP. These results suggest the NO
pathway or bioavailability could be impaired once Ach releases
NO by the endothelial cell, while the SNP provides an inorganic
source of NO [63,64].

Superoxide anions (O$_2^-$) are largely responsible for altering the
bioavailability of NO by forming peroxynitrite (ONOO$^-$) [65].
Several enzymatic sources in blood vessels may produce ROS.
NADPH oxidase complex is one of the most important of these
sources and may be the largest producer of O$_2^-$ in the vascular
wall [66]. Thus, we performed additional experiments in vessels in
presence of NADPH oxidase inhibitor (apocynin). When the
NADPH oxidase was inhibited by pre-incubation with apocynin,
the vasorelaxation of supplemented animals improved and
returned to similar vascular response to control group. Therefore,
VD supplementation could be the responsible for the increased
source of the ROS by NADPH oxidase complex and decreasing the
NO bioavailability. This decreased NO bioavailability leads to
impaired vascular relaxation, which may be a mechanism of
increased arterial systolic pressure in this model.

### Table 3. Summary of pD$_2$ and Maximal Response values.

| Variable | C (n = 20) | VD3 (n = 19) | VD10 (n = 21) | p |
|----------|-----------|-------------|-------------|---|
| Phe E$^+$(%KCl) | | | | |
| pD$_2$ | 7.9±0.8 (5) | 8.8±0.6 $^{*}$ (6) | 8.1±0.3 (6) | 0.041 |
| Maximal Response | 157.0±37.5 (5) | 250.3±92.3 (6) | 197.0±49.5 (6) | 0.095 |
| Phe E$^-$ (%KCl) | | | | |
| pD$_2$ | 7.4±0.5 (5) | 7.7±0.2 (5) | 7.3±0.4 (6) | 0.236 |
| Maximal Response | 148.7±41.9 (5) | 166.0±33.4 (5) | 138.4±33.6 (6) | 0.466 |
| Ach (%) | | | | |
| pD$_2$ | 7.5±0.6 (9) | 7.8±0.9 (8) | 7.0±0.3 (7) | 0.101 |
| Maximal Response | 97.5 (71.7–100.1) (9) | 90.6 (75.2–95.9) (8) | 60.9 (55.6–80.1$^{*}$) (7) | 0.023 |
| Ach + apocynin (%) | | | | |
| pD$_2$ | 7.2±1.0 (4) | 8.7±0.4 $^{*}$ (5) | 8.4±0.5 $^{*}$ (6) | 0.013 |
| Maximal Response | 101.1±1.4 (4) | 96.4±5.3 (5) | 94.8±6.6 (6) | 0.213 |
| SNP (%) | | | | |
| pD$_2$ | 7.4±0.5 (5) | 6.9±0.6 (5) | 7.1±0.5 (5) | 0.416 |
| Maximal Response | 120.7±13.9 (6) | 96.1±15.3 $^{*}$ (5) | 92.3±6.5 $^{*}$ (5) | 0.005 |
| SNP + apocynin (%) | | | | |
| pD$_2$ | 9.8±0.3 (4) | 9.4±0.6 (5) | 9.2±0.5 (5) | 0.198 |
| Maximal response | 102.2 (100.4–121.3) (4) | 102.0 (100.0–105.2) (5) | 105.0 (103.2–108.0) (5) | 0.382 |

Data are expressed as mean ± standard deviation of mean or median with 25 and 75 percentiles; numbers in parentheses indicate the numbers of animals included in each experimental group. C: control group (no supplementation with vitamin D); VD3: supplemented with 3,000 IU VD/kg of chow; VD10: supplemented with 10,000 IU VD/kg of chow. pD$_2$: indicates -log EC$_{50}$ (the concentration of agonist producing half-maximal response); Phe: phenylephrine; E$^+$: endothelium-intact vessels; E$^-$: endothelium-denuded vessels; Ach: acetylcholine; SNP: sodium nitroprusside. * p<0.05 versus control group.

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Increased arterial pressure is also associated with structural and mechanical alterations in both resistance and conduit arteries [67]. The maintenance proper structural geometry, mechanical properties and function of vessels are dependent on the balanced composition of the extracellular components matrix (ECM) [68,69]. We showed that supplemented animals did not present alterations in media cross-sectional area, media thickness and lumen diameter. However, they showed alterations in ECM. The collagen content was higher, and the elastin content was lower in the animals supplemented with highest VD dose. Therefore, the ratio collagen/elastin was elevated in these animals. However, no difference was observed in elasticity (yield point) and vascular stiffness. It can be speculated that occurred production of other collagen type or architecture rearranged in order to preserve the integrity and the mechanical properties of the vessel wall [70,71].

The ECM metabolism is regulated for metalloproteinases (MMPs), which are Zn21- and Ca21-dependent proteolytic enzymes [72]. Several different MMPs are present in the vasculature. These MMPs include MMP-2 and MMP-9, which play an important role in vascular remodeling [73,74,75]. In our study, MMP2 activity was higher in the supplemented animals compared to control. Increased MMP-2 activity is associated with increased deposition of collagen, alterations in ECM architecture or ECM attachments [76], systemic arterial stiffness [77] and modulation of vascular contractility and relaxation [78,79], thereby promoting vasoconstriction. Furthermore, MMP-2 activities are also associated with the destruction of the elastic lamina of arteries [80]. In situ studies showed gelatinolytic activity in tissue sections and strong MMP-2 immunostaining along the inner elastic lamina up to the lamina break [81]. Clinical and experimental studies have reported increased expression and activity of MMPs, particularly MMP-2 in the vascular tissues in animal hypertension models [51,82]. Therefore, the alterations observed in ECM of supplemented animals may be associated with alterations in MMPs.

Studies have shown that increased oxidative stress and reduction in NO bioavailability both contribute to increased MMP-mediated vascular remodeling and resulting vascular pathologies [69,83,84]. In addition, during this process, ONOO−-generated activates latent MMPs. These processes lead to the degradation of ECM components elastin and collagen. However, because the turnover of collagen is fast, more collagen is placed on the outer interstitial and inner medial layers of the aorta wall [84]. This placement may explain the relation between the higher collagen content observed in the VD10 group, which also had increased MMP-2 activity.

Table 4. Histological data of the aorta.

| Variable       | C            | VD3          | VD10         | p            |
|----------------|--------------|--------------|--------------|--------------|
| CSA (µm²)      | 7519±1555    | 6484±1129    | 7676±1642    | 0.370        |
| (6)            | (6)          | (3)          |              |              |
| M (µm)         | 13.3 (13.1–16.1) | 12.0 (10.5–12.4) | 12.9 (12.3–13.6) | 0.235        |
| (6)            | (6)          | (3)          |              |              |
| L (µm)         | 157±14.9     | 155±13.3     | 175±28.1     | 0.269        |
| (6)            | (6)          | (3)          |              |              |
| M/L            | 0.087 (0.083–0.096) | 0.072 (0.068–0.077) | 0.070 (0.070–0.081) | 0.130        |
| (6)            | (6)          | (3)          |              |              |
| Collagen (%)   | 0.26±0.03    | 0.22±0.03    | 0.29±0.03*   | <0.001       |
| (8)            | (8)          | (8)          |              |              |
| Elastin (%)    | 0.35±0.04    | 0.35±0.03    | 0.30±0.03*   | 0.015        |
| (9)            | (8)          | (9)          |              |              |
| Collagen/elastin | 0.76±0.10  | 0.64±0.11    | 0.95±0.19*   | <0.001       |
| (8)            | (8)          | (7)          |              |              |

Data are expressed as mean ± standard deviation of mean or median with 25 and 75 percentiles; numbers in parentheses indicate the numbers of animals included in each experimental group. C: control group (no supplementation with vitamin D); VD3: supplemented with 3,000 IU VD/kg of chow; VD10: supplemented with 10,000 IU VD/kg of chow. CSA: media cross-sectional area; M: media thickness; L: lumen diameter. * p < 0.05 versus control group; # p < 0.05 versus VD3 group.

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Figure 1. Elastin content in the aortic sections and fragmentation of elastic fibers in the VD10 group. Photographs of aortic samples (400×) stained by Calleja. C: control group (no supplementation with vitamin D); VD3: supplemented with 3,000 IU VD/kg of chow; VD10: supplemented with 10,000 IU VD/kg of chow.

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The higher collagen content in the group VD10 may also have prevented the increase in aortic contractility in these animals. Study has shown that the increased bulk collagen interposed between the smooth muscle cells reduced the force generated by the smooth muscle cells. Another possibility is that the attachments between smooth muscle cells and extracellular matrix are altered and influence the maximal tension generated [85]. Finally, VD supplementation in normotensive rats led to increased systolic blood pressure, but the mechanisms involved may be different, depending on the dose used. In the VD10 group, the dose was related to impaired vascular relaxation and changes in ECM. While in the VD3 group, the dose was related to increased vascular contractility and alterations of the aortic mechanical properties.

Several studies showed that VD exerts a biphasic “dose response” curve on cardiovascular physiopathology with deleterious consequences not only of VD deficiency but also of VD excess [86,87]. Both VD deficiency [88,89,90,91,92,93,94] and high doses of VD [95,96,97,98] can lead to structure and functional vascular alterations and hypertension. In addition, the VD deficiency is associated with marked increase in renin activity [99]. On the other hand, in hypertension models the VD presented antihypertensive effect. The antihypertensive mechanisms include the lower regulator for rennin, protects the vascular function and the inhibition of vascular smooth muscular cell proliferation and growth [31,99,100,101,102,103].

In conclusion, our data suggest that the higher arterial pressure in normotensive rats after VD supplementation were caused by aortic alterations in function and structure. NO bioavailability and ROS production may also play an important role in this increased pressure.

Author Contributions
Conceived and designed the experiments: PPS FMF LAMZ SARP. Performed the experiments: PPS BPMR AFG RGJ TBN MABS SBAC. Analyzed the data: PPS RGJ TBN RCT LAMZ SARP. Contributed reagents/materials/analysis tools: PPS FMF RCT LAMZ SARP. Wrote the paper: PPS RCT LAMZ SARP.

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Table 5. Data on the vascular mechanical properties and Metalloproteinase-2 and -9 activities by gelatin zymography.

| Variable                          | C               | VD3                          | VD10                          | P      |
|-----------------------------------|-----------------|------------------------------|-------------------------------|--------|
| Yield point (N)                   | 4.2 (3.8–5.5)   | 2.4 (2.1–2.9)                | 3.8 (2.1–5.1)                 | 0.009  |
| Stiffness (N/mm)                  | 1.4 ± 0.4 (10)  | 1.2 ± 0.3 (9)                | 1.1 ± 0.2 (9)                 | 0.209  |
| Failure load (N)                  | 5.2 ± 1.5 (10)  | 4.2 ± 1.6 (9)                | 4.6 ± 1.1 (9)                 | 0.394  |
| Active/inactive MMP-2 ( Arbitrary units) | 1.64 (0.72–2.06) (11) | 1.19 (1.04–1.48) (11) | 2.37 (1.49–3.76) (9) | 0.041  |
| Active/inactive MMP-9 ( Arbitrary units) | 5.90 (1.46–9.84) (10) | 2.68 (1.69–5.02) (10) | 4.33 (1.92–11.94) (9) | 0.772  |

Data are expressed as mean ± standard deviation of mean or median with 25 and 75 percentiles, numbers in parentheses indicate the numbers of animals included in each experimental group. C: control group (no supplementation with vitamin D); VD3: supplemented with 3,000 IU VD/kg of chow; VD10: supplemented with 10,000 IU VD/kg of chow. * p<0.05 versus control group; # p<0.05 versus VD3 group.

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