Impact of the Length of Vitamin D Deficiency on Cardiac Remodeling

Assalin et al: Vitamin D Deficiency and Cardiac Remodeling

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Abstract

Background—This study was aimed to evaluate the influence of vitamin D (VD) deficiency on cardiac metabolism, morphology and function. Thus, we investigated the relationship of these changes with the length of the nutrient restriction.

Methods and Results—Male weanling Wistar rats were allocated into four groups: C2(n=24), animals were fed an AIN-93G diet with 1,000 IU VD/kg of chow and were kept under fluorescent light for 2 months; D2(n=22), animals were fed a VD – deficient AIN-93G diet and were kept under incandescent light for 2 months; C4(n=21) animals were kept in the same conditions of C2 for 4 months; D4(n=23) animals were kept in the same conditions of D2 for 4 months. Biochemical analyses showed lower beta-hydroxyacyl coenzyme-A dehydrogenase activity and higher lactate dehydrogenase (LDH) activity in VD deficient animals. Furthermore, VD deficiency was related to increased cytokines release, oxidative stress, apoptosis and fibrosis. Echocardiographic data showed left ventricular (LV) hypertrophy and lower fractional shortening and ejection fraction in VD deficient animals. Difference became evident in the LDH activity, LV weight, right ventricle weight, and LV mass after 4 months of VD deficiency.

Conclusions—Our data indicate that VD deficiency is associated to energetic metabolic changes, cardiac inflammation, oxidative stress, fibrosis and apoptosis, cardiac hypertrophy, left chambers alterations and systolic dysfunction. Furthermore, length of the restriction influenced these cardiac changes.

Key Words: vitamin D deficiency; cardiac remodeling; oxidative stress
The classic effects of vitamin D (VD) are pivotal to bone development, growth, mineralization, maintenance of skeletal integrity and calcium and phosphorus homeostasis\(^1\). However, in recent years, a wide variety of extra-skeletal conditions have also been associated with VD deficiency (VDD)\(^1,2\), including the regulation of cell proliferation/differentiation, modulation of the immune system, influence on pancreatic \(\beta\)-cell function and regulation of cardiac contractility and hypertrophy\(^3\).

Epidemiological studies describe an association between VDD and several cardiovascular diseases, suggesting that VDD has a negative association with survival\(^4,5\).

Experimental studies also indicated that a VD deficient diet is related to changes in contractile function in isolated myocytes, higher systolic blood pressure, myocardial relaxation, fibrosis and hypertrophy\(^6-8\). Additionally, murine cardiomyocytes isolated from VD receptor knockout mice show accelerated contraction and relaxation rates and cellular hypertrophy of the heart myofibrils\(^9\). Some of these cardiac changes associated with VDD are characteristic of cardiac remodeling.

Cardiac remodeling may be defined as changes in the size, geometry, shape, composition, and function of the heart in response to diverse stimuli. At first, ventricular remodeling is a compensatory process, and these morphological adaptations are essential to preserving cardiac function. However, chronic ventricular remodeling is recognized as a significant pathologic process that results in progressive ventricular dysfunction and the eventual clinical presentation of heart failure or sudden death\(^10,11\).

Despite several studies showing that a VD deficient diet induces cardiac changes, the influence of the length of VD restriction on cardiac tissue is unknown. In addition, the mechanisms involved in this process remain to be elucidated. Given that ventricular remodeling is modulated by the degree of injury, we hypothesized that a longer exposure to a
VD deficient diet might be associated with greater degrees of cardiac morphological and functional alterations.

The aim of the present study was to assess the cardiac effects of VDD in an animal model in terms of metabolism, inflammation, oxidative stress, morphology and function. Additionally, because the extent of cardiac remodeling has been linked to the magnitude of injury, the second aim of the present study was to evaluate the consequences of VDD at two time points: two and four months after initiation of a VD deficient diet.

Methods

Experimental protocol: Male weanling Wistar rats were housed and taken care of in accordance with the National Institute of Health’s Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Ethics Committee of the Botucatu School of Medicine-UNESP, Brazil (protocol No. 693/2008). The animals were randomly allocated into four groups (C2, D2, C4 and D4) and raised on different diets for two or four months (Figure 1). The animals in group C2(n=24) were fed an AIN-93G diet with 1,000 IU VD/kg of chow and were kept under fluorescent lights for 2 months. The animals in group D2(n=22) were fed a VD – deficient AIN-93G diet that contained 0.7% calcium and 0.3% phosphorus and were kept under incandescent lights for 2 months to prevent VD cutaneous synthesis. The C4(n=21) animals were kept in the same conditions of C2 for 4 months. The D4(n=23) animals were kept in the same conditions of D2 for 4 months. The daily food consumption by individual rats was measured and body weight was measured weekly. After the period of treatment, each animal was submitted to an echocardiographic study and followed by euthanasia.

Tail systolic pressure: Systolic arterial pressure measurement was performed two weeks before euthanasia. A tail plethysmograph was used as described previously. 12
**Echocardiographic study:** All of the animals were evaluated with a transthoracic echocardiograph exam, according to a previously described method\(^{13}\).

**Morphometric analysis:** Following echocardiographic study, the animals were euthanized with large dose of pentobarbital, and their hearts and tibia were removed. The right and left ventricles (LV), and the atrium were dissected, separated, and weighed. Transverse sections of LV were fixed in 10% buffered formalin and embedded in paraffin. Five-micron-thick sections were stained with hematoxylin – eosin (HE) or picrosirius red. The myocyte cross-sectional area and interstitial collagen volume fraction were measured as previously described\(^{14}\).

**Connexin 43 and caspase 3 immunohistochemistry:** Histological LV sections were processed as previously described for immunohistochemistry evaluation of connexin 43 (Connexin 43: polyclonal anti-rabbit connexin-43, GJA1, Abcam, Cambridge, MA, USA) and caspase 3 (Caspase 3: cleaved caspase -3 (Asp175) (5A1) rabbit mAb)\(^{12}\).

**Vitamin D determination:** Serum concentrations of 25-hydroxycholecalciferol (25[OH]D\(_3\)) were measured by high performance liquid chromatography as described by Asknes\(^{15}\).

**Biochemical analysis:** Serum concentrations of calcium and phosphorus were measured using the arsenazo III and colorimetric methods, respectively (test kit Labor Lab, São Paulo, Brazil). Serum concentrations of parathyroid hormone (PTH) were measured according to the PTH (Intact, Rat) Elisa Kit instructions (Alpco\(^{\text{TM}}\) Immunoassays, Salem, NH, USA).

**Cytokine production:** The production of tumor necrosis factor alpha (TNF-\(\alpha\)), interferon gamma (IFN-\(\gamma\)) and interleukin 10 (IL-10) was evaluated by ELISA according to the manufacturer instructions (R & D Systems, Minneapolis, MN, USA)\(^{16}\).
Cardiac lipid hydroperoxide, antioxidant enzymes and energy metabolism analysis: Total protein, lipid hydroperoxide (LH), glutathione peroxidase (GSHPx, E.C.1.11.1.9), superoxide dismutase (SOD, E.C.1.15.1.1) and catalase (CAT, E.C.1.11.1.6) activities were assessed as previously specified\textsuperscript{17-19}. Cardiac energy metabolism was assessed by beta-hydroxyacyl coenzyme-A dehydrogenase (OHADH, E.C.1.1.1.35.) and lactate dehydrogenase (LDH, E.C.1.1.1.27) activities\textsuperscript{20}.

Western blotting analysis: Nuclear and cytoplasmic protein extraction from the LV samples was performed with the NE-PER\textsuperscript{®} Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology, Rockford, USA). Nuclear extracts were used for Nrf2 detection (Nrf2 (C-20, rabbit IgG (Santa Cruz Biotechnology, Inc, Europe)). The cytoplasmic extract was used for heme oxygenase 1 ([HO-1-1] to heme oxygenase 1; ab13248; Abcam Inc, Cambridge) and glutathione peroxidase (GPx Rabbit polyclonal to glutathione peroxidase 1; ab22604, Abcam Inc, Cambridge) detection.

Statistical analysis: The data are reported as the means ± SEM. The results were tested for normality (Kolmogorov-Smirnov test) and equal variance prior to the statistical analyses and all the data passed these tests. Also, the 4 groups of animals were independent. Therefore, data were analyzed by a 2-way ANOVA. One factor is the VD content in the diet, and the other factor is the length of the nutrient restriction. The first factor was called “Diet” and the second factor is “time”. When an interaction was found to be significant, the means were compared using Tukey post-hoc analysis. In the present study, there was no interest in analyzing the differences observed in the factor length of the nutrient restriction because this is an alteration related to normal rat development.

An ANCOVA test was also used to test the PTH and calcium influence on the cardiac variables.
The differences were considered statistically significant if p<0.05 and for some results the 95% confidence interval are presented. The statistical analyses were performed using Systat for Windows v12.0 (Systat Software, Inc. San Jose, CA).

Results

The animal model used in the present study was effective for inducing nutritional VDD because the treated animals showed lower 25(OH)D₃ levels and higher PTH levels. An interaction was found when analyzing PTH (Table 1). The PTH values were higher in VD deficient animals both at 2 months (D2 > C2, p=0.002 and 95%CI= -838 to -157) and at 4 months (D4 > C4, p<0.001 and 95%CI= -1298 to -595) (Figure 2). The PTH values increased with time (D4 > D2, p=0.024 and 95%CI= -741 to -39). No interaction was observed for 25(OH)D₃ or calcium levels. Lower values were found in deficient vitamin D diet (DVD) animals for 25(OH)D₃ (Figure 3) (p<0.001 and 95%CI= 245 to 336) and calcium levels (Table 1) (p<0.001 and 95%CI= 0.72 to 4.80) compared with sufficient vitamin D animals (SVD). An ANCOVA test was performed to check the influence of serum PTH and calcium levels on all of the cardiac variables. No statistically significant influence was observed for either covariate (data not shown).

No interaction between the factors and no significant differences related to VD content in the diet factor were observed for final body weight, tibia length, food consumption, phosphorus levels or systolic pressure, as shown in Table 1.

Biochemical analyses of cardiac energy metabolism are shown in Figure 4. An interaction was observed for OHADH activity, which was lower in DVD animals at 2 months (D2 < C2, p=0.001 and 95%CI= 9.35 to 41.80) and at 4 months (D4 < C4, p<0.001 and 95%CI= 61.7 to 94.1). Furthermore, D4 animals showed lower enzyme activity than D2 animals (p<0.001 and 95%CI= 13.2 to 45.6). An interaction was also observed for LDH
activity, which was higher in DVD animals at 4 months (D4 > C4, p<0.001 and 95%CI= -198 to -90).

No interaction between factors was observed for cardiac inflammation. However, differences were observed for the factor of VD content in the diet. TNF-α (p=0.013 and 95%CI= -4.20 to -0.67) and INF-γ (p=0.031 and 95%CI= -10.14 to -0.71) were higher in DVD animals than in SVD animals, showing that VDD was related to increased cytokine release (Table 2).

Table 3 shows data related to cardiac oxidative stress. No interaction was observed for all variables. However, a statistical significance was found for the factor of VD content in the diet. The LH levels (p<0.001 and 95%CI= -154 to -89), Nrf-2 (p=0.002 and 95%CI= -0.32 to -0.08), HO-1 (p=0.012 and 95%CI= -0.60 to -0.09) and GPx (p=0.029 and 95%CI= -0.75 to -0.05) amounts were higher in DVD animals than in SVD animals. Catalase (p=0.011 and 95%CI= 0.11 to 0.72) and GSHPx (p<0.001 and 95%CI= 7.74 to 22.20) activity were lower in DVD animals.

The data describing the cardiac caspase 3, connexin 43 and cardiac morphometric analyses are listed in Table 4. An interaction between factors was observed for LV weight and RV weight. LV weight and RV weight were higher in DVD animals at 4 months (D4 > C4, LV weight - p=0.022 and 95%CI= -0.037 to -0.002 and RV weight - p=0.020 and 95%CI= -1.40 to -0.09). Furthermore, LV weight was higher in C4 and D4 animals than in C2 (p=0.049 and 95%CI= -0.035 to 0.000) and D2 (p<0.001 and 95%CI= -0.060 to 0.025) animals, respectively. A difference for the factor of VD content in the diet was observed for caspase 3 (p=0.040 and 95%CI= -24.7 to -0.67) and interstitial collagen (p=0.046 and 95%CI= -2.17 to -0.06). DVD animals had higher numbers of labeled caspase 3 cells and increased interstitial collagen compared with SVD animals, increased apoptosis and fibrosis due to VDD.
The echocardiographic data are presented in Table 5. Interactions were found when analyzing the right atrium area (RAA) and the left ventricle mass (LVM). RAA and LVM were higher in DVD animals at 4 months (D4 > C4, RAA - p=0.045 and 95%CI= -0.052 to 0.000 and LVM - p=0.003 and 95%CI= -239 to -37). Differences were also observed for the VD content in the diet factor. The left ventricle end-diastolic diameter (LVDD - p=0.006 and 95%CI= -0.108 to -0.019), left ventricle end-systolic diameter (LVSD - p=0.002 and 95%CI= -0.138 to -0.032) and left atrium (LA - p<0.001 and 95%CI= -0.012 to -0.004) were increased in DVD animals compared with SVD animals. Fractional shortening (FS - p=0.018 and 95%CI= 0.006 to 0.065) and the ejection fraction (EF - p=0.023 and 95%CI= 0.002 to 0.029) were lower in DVD animals compared with SVD animals. No differences were observed for other echocardiographic variables.

**Discussion**

25[OH]D is the primary storage form of VD and the metabolite that best represents the state of VD sufficiency. The characterization of the deficient state was obtained by measuring plasma levels of 25[OH]D, and the experimental model used in the present study proved effective at inducing a nutritional status characterized as VDD in rats. Several studies show that VDD is associated with hypocalcaemia and secondary hyperparathyroidism. Calcium and PTH can directly influence cardiac function. Certain studies that have utilized the VD deficient diet model increase the amount of calcium and phosphorus in the chow to maintain calcium, phosphorus and PTH within the normal range. In the present study, hypocalcaemia and hyperparathyroidism were observed despite the increase in the maximal amount of calcium and phosphorus supplementation in the rat chow. Therefore, to adjust for the influence of the serum calcium and PTH levels an ANCOVA test was performed. We observed no influence of calcium and PTH on the cardiac variables.
These results corroborate other studies showing that the cardiac alterations due to VDD occur independently of calcium and PTH^{3,27,28}.

It is well-known that VD plays a direct or indirect role on multiple genes in different organs and tissues. Therefore, some studies have shown that VDD can influence energetic metabolism^{29}, inflammation^{30-32} and oxidative stress^{33}. These changes are part of the cardiac remodeling process.

Heart tissue is capable of meeting its energy needs through the oxidation of fatty acids, glucose, lactate and other oxidizable substrates. Under normal physiological conditions in the heart, fatty acids are the preferred source of energy generated via β-oxidation^{34}. The increase in the OHADH in C4 group suggests a better fatty acid oxidation metabolism with a reduced utilization of glucose oxidation as fuel for the heart. Disturbances in myocardial substrate utilization have adverse effects in the failing myocardium^{35}, and shifting the substrate preference of the heart away from fatty acids towards carbohydrate oxidation can improve pump function and slow the progression of heart failure^{36}. In the present study, we hypothesize that VDD have a change in substrate utilization. Activation of glycolytic pathway and reduced β-oxidation is a marker of cardiac remodeling and heart failure.

In different heart failure models, cytokine production can modulate cardiac remodeling. Indeed, increased INF-γ and especially TNF-α levels are associated with left ventricle dysfunction, activation of fetal gene programming, apoptosis, hypertrophy and fibrosis^{37}. In the present study, the remodeling process in VD deficient animals was associated with altered cardiac levels of INF-γ and TNF-α. In fact, this result was expected because VD has a role in the modulation of immune and inflammatory responses^{31}. In the adaptative immune system, 1,25(OH)_2D inhibits the proliferation of T helper 1 (Th1) cells and thus limits cytokine production by these cells. In the absence of VD, Th1 cell inhibition does not occur, and INF-γ and TNF-α levels increase, which was expected as observed in our
study. Conversely, 1,25(OH)\textsubscript{2}D induces T helper 2 (Th2) cytokines and regulatory T cells (Treg)\textsuperscript{30,32}. Therefore, without no Th2 stimulation, it is expected to not alter IL-10 levels. Thus, the loss of adaptive immune response suppression commonly caused by VDD could be due to the increase of the inflammatory markers observed in this model.

It is known that alterations of the metabolic pathway and activation of immune cells may produce free radicals\textsuperscript{38}. Oxidative stress results from an oxidant/antioxidant imbalance, which is an excess of oxidants relative to antioxidant capacity\textsuperscript{38}. The antioxidant enzymes are the first line of defense against oxidative stress, particularly CAT, SOD and GSHPx\textsuperscript{39}. VDD was related to higher vascular oxidative stress\textsuperscript{33}. In the present study, VDD was associated not only with increased reactive oxygen species (ROS) in the cardiac tissue, as shown by the higher LH, but also with reduced activity of CAT and GSHPx. It is important to consider a possible mechanism to compensate the increased oxidative stress that occurs through the Nrf-2 pathway.

Nrf-2 is a transcriptional factor that binds and activates the antioxidant response element (ARE) in the promoters of many antioxidant and detoxification genes\textsuperscript{40}. In the inactive form, Nrf-2 is bound to the protein Keap1 in the cytoplasm. Lipid hydroperoxides can promote conformational changes of the Keap1 and Nrf-2 actives sites\textsuperscript{41}, leading to release of Keap1 and migration of Nrf-2 to the nucleus. Many antioxidant genes, such as GPx and HO-1, contain an ARE in the promoter\textsuperscript{41}. Thus, in the present study we hypothesize that VDD could be related to higher expression of nuclear Nrf-2 and cytoplasmic GPx and HO-1 in cardiomyocytes, which was an attempt of the cardiac cells to improve the enzymatic antioxidant defense system.

Oxidative stress and proinflammatory cytokines are related to increased fibrosis and apoptosis. Myocardial fibroblast cells activated by proinflammatory cytokines and oxidative stress become highly proliferative and invasive and actively remodel the cardiac interstitium.
In addition, cardiac cell apoptosis is mediated through several signaling systems that involve ROS and inflammatory cytokines. The end point is the activation of the caspase pathway\textsuperscript{42}. In the present study, VDD was associated with abnormal interstitial collagen accumulation and with higher apoptosis as assessed by caspase 3. The fibrosis and cell loss that occurs through apoptosis contributes to morphological alterations and also plays an important role in the impairment of cardiac performance.

Considering the morphological and functional consequences of VDD, in the present study, we observed cardiac hypertrophy and enlargement of the left chambers with preserved relative wall thickness featuring eccentric remodeling\textsuperscript{43}. It is well accepted that the ventricular remodeling process plays a fundamental role in the pathophysiology of ventricular dysfunction. In accordance with this concept, VDD was associated with an impaired systolic function, which was characterized by reduced fractional shortening and ejection fraction.

As mentioned, the present study also aimed to analyze the influence of length of nutrient restriction on the cardiac variables. Importantly, the length of VDD influenced certain changes. Although PTH levels and OHADH activity were significantly different after 2 months of deficient VD diet use, this difference became more evident after 4 months. These findings show that the VDD was an ongoing process. In relation to LDH activity, LV, RV, RAA and LVM, no significant differences were detected after 2 months of deficient VD diet use. However, after 4 months of VDD, the difference became evident. Thus, it was possible to analyze the worsening evolution of the variables over time and conclude that, especially for variables indicative of cardiac hypertrophy, longer VDD highlights changes that are not observed with shorter times.

Finally, we should considerer the potential limitations of this study. It is well accepted that the investigation of diastolic function is a challenger. Indeed, despite expected in the present analysis, diastolic dysfunction was not observed. It seems to be related to the
methodology in evaluation of diastolic function by conventional echocardiogram. In addition, another limitation to measure diastolic function in this model is the high cardiac frequency in rats. Unfortunately, our study did not use tissue echocardiogram, which is the most sensitive non-invasive method for the assessment of diastolic function. Thus, the effects of VDD on diastolic function remain to be elucidated.

In conclusion, VDD in a restriction diet model is an ongoing process that is associated with cardiac remodeling, and the duration of this deficiency determines the intensity of the alterations.

Sources of Funding

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Disclosures

None.

References

Table 1. Body weight, tibial length, food ingestion, systolic pressure and biochemical analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups C2</th>
<th>C4</th>
<th>D2</th>
<th>D4</th>
<th>Diet</th>
<th>Time</th>
<th>Diet x Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>375.2±9.0</td>
<td>461.6±9.3</td>
<td>371.3±8.6</td>
<td>484.7±8.6</td>
<td>0.285</td>
<td>&lt;0.001</td>
<td>0.134</td>
</tr>
<tr>
<td>Tibial length (mm)</td>
<td>39.4±0.3</td>
<td>41.9±0.3</td>
<td>39.6±0.3</td>
<td>41.9±0.3</td>
<td>0.818</td>
<td>&lt;0.001</td>
<td>0.701</td>
</tr>
<tr>
<td>Food consumption (g/day)</td>
<td>16.2±0.3</td>
<td>18.3±0.3</td>
<td>16.0±0.3</td>
<td>18.4±0.3</td>
<td>0.863</td>
<td>&lt;0.001</td>
<td>0.615</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>110.8±4.2</td>
<td>137.5±4.4</td>
<td>113.6±4.1</td>
<td>131.8±4.3</td>
<td>0.735</td>
<td>&lt;0.001</td>
<td>0.315</td>
</tr>
<tr>
<td>Ca (mg/dl)</td>
<td>10.7±0.3</td>
<td>9.7±0.3</td>
<td>9.9±0.3</td>
<td>9.7±0.3</td>
<td>&lt;0.001</td>
<td>0.079</td>
<td>0.304</td>
</tr>
<tr>
<td>P (mg/dl)</td>
<td>10.2±0.4</td>
<td>8.8±0.4</td>
<td>9.9±0.4</td>
<td>8.7±0.4</td>
<td>0.501</td>
<td>0.028</td>
<td>0.767</td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D. BW = body weight; Ca = serum calcium; P = serum phosphorus. Diet (factor vitamin D content in the diet) = p-value of the vitamin D content in the diet effect; Time (factor length of the nutrient restriction) = p-value of the time effect; Diet x time = p-value of the interaction.
Table 2. Cardiac inflammation

<table>
<thead>
<tr>
<th>Variable</th>
<th>C2</th>
<th>D2</th>
<th>C4</th>
<th>D4</th>
<th>Diet</th>
<th>Time</th>
<th>Diet x Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 (pg/mg)</td>
<td>13.4±3.5</td>
<td>10.9±2.7</td>
<td>8.6±3.0</td>
<td>15.5±3.0</td>
<td>0.490</td>
<td>0.974</td>
<td>0.151</td>
</tr>
<tr>
<td>TNF-α (pg/mg)</td>
<td>2.1±1.0</td>
<td>4.9±0.8</td>
<td>3.1±0.7</td>
<td>5.2±0.8</td>
<td>0.013</td>
<td>0.471</td>
<td>0.664</td>
</tr>
<tr>
<td>INF-γ (pg/mg)</td>
<td>3.6±2.8</td>
<td>9.7±2.2</td>
<td>6.2±2.0</td>
<td>10.9±2.2</td>
<td>0.031</td>
<td>0.423</td>
<td>0.780</td>
</tr>
<tr>
<td>ICAM-1 (pg/mg)</td>
<td>52.0±8.1</td>
<td>61.3±6.3</td>
<td>54.4±7.0</td>
<td>58.6±6.3</td>
<td>0.351</td>
<td>0.979</td>
<td>0.719</td>
</tr>
</tbody>
</table>

The dates are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D. IL-10 = interleukin 10; TNF-α = tumor necrosis factor-alpha; INF-γ = interferon-gamma; ICAM-1 = intercellular adhesion molecule. Diet (factor vitamin D content in the diet) = p-value of the vitamin D content in the diet effect; Time (factor length of the nutrient restriction) = p-value of the time effect; Diet x time = p-value of the interaction.
Table 3. Cardiac oxidative stress: protein activity and quantification

<table>
<thead>
<tr>
<th>Variable</th>
<th>C2</th>
<th>D2</th>
<th>C4</th>
<th>D4</th>
<th>Diet</th>
<th>Time</th>
<th>Diet x Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (nmol/g tissue)</td>
<td>160.7±15.6</td>
<td>289.4±15.6</td>
<td>143.5±15.6</td>
<td>257.4±15.6</td>
<td>&lt;0.001</td>
<td>0.131</td>
<td>0.642</td>
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<tr>
<td>CAT (μmol/mg protein)</td>
<td>1.6±0.2</td>
<td>1.0±0.2</td>
<td>1.4±0.2</td>
<td>1.1±0.2</td>
<td>0.011</td>
<td>0.553</td>
<td>0.253</td>
</tr>
<tr>
<td>GSHPx (nmol/mg tissue)</td>
<td>59.1±3.5</td>
<td>37.6±3.5</td>
<td>45.5±3.5</td>
<td>37.1±3.5</td>
<td>&lt;0.001</td>
<td>0.054</td>
<td>0.075</td>
</tr>
<tr>
<td>SOD (nmol/mg protein)</td>
<td>13.1±1.4</td>
<td>13.3±1.4</td>
<td>11.9±1.4</td>
<td>8.7±1.4</td>
<td>0.303</td>
<td>0.055</td>
<td>0.255</td>
</tr>
<tr>
<td>Nrf-2</td>
<td>0.69±0.06</td>
<td>0.87±0.06</td>
<td>0.78±0.06</td>
<td>1.00±0.06</td>
<td>0.002</td>
<td>0.063</td>
<td>0.735</td>
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<tr>
<td>HO-1</td>
<td>1.49±0.15</td>
<td>1.74±0.11</td>
<td>1.79±0.12</td>
<td>2.18±0.11</td>
<td>0.020</td>
<td>0.008</td>
<td>0.586</td>
</tr>
<tr>
<td>GPx</td>
<td>1.34±0.19</td>
<td>1.72±0.16</td>
<td>1.24±0.21</td>
<td>1.77±0.16</td>
<td>0.019</td>
<td>0.877</td>
<td>0.669</td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D. LH = lipid hydroperoxide; CAT = catalase activity; GSHPx = glutathione peroxidase activity; SOD = superoxide dismutase activity; Nrf-2 = nuclear erythroid factor2; HO-1 = heme oxygenase 1; GPx = glutathione peroxidase. Diet (factor vitamin D content in the diet) = p-value of the vitamin D content in the diet effect; Time (factor length of the nutrient restriction) = p-value of the time effect; Diet x time = p-value of the interaction.
Table 4. Morphometric and immunohistochemistry data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>p-value</th>
<th>Diet</th>
<th>Time</th>
<th>Diet x Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV weight/tibia</td>
<td>0.20±0.01²</td>
<td>0.19±0.00³</td>
<td>0.22±0.00²</td>
<td>0.24±0.01³</td>
<td>0.151</td>
</tr>
<tr>
<td>RV weight/tibia</td>
<td>5.56±0.17⁴</td>
<td>5.43±0.18</td>
<td>5.05±0.18</td>
<td>5.79±0.17³</td>
<td>0.087</td>
</tr>
<tr>
<td>Atrium /tibia</td>
<td>0.88±0.08</td>
<td>0.82±0.08</td>
<td>0.79±0.08</td>
<td>0.98±0.08</td>
<td>0.446</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>13.4±5.5</td>
<td>29.1±5.5</td>
<td>15.1±6.1</td>
<td>24.8±5.5</td>
<td>0.040</td>
</tr>
<tr>
<td>Localization connexin 43</td>
<td>2.2±0.2</td>
<td>2.1±0.2</td>
<td>2.0±0.2</td>
<td>2.5±0.2</td>
<td>0.392</td>
</tr>
<tr>
<td>Intensity connexin 43</td>
<td>2.5±0.2</td>
<td>2.7±0.1</td>
<td>2.4±0.1</td>
<td>2.5±0.2</td>
<td>0.291</td>
</tr>
<tr>
<td>MCA (µm²)</td>
<td>215.9±15.7</td>
<td>236.6±14.6</td>
<td>275.8±14.6</td>
<td>283.4±15.7</td>
<td>0.359</td>
</tr>
<tr>
<td>Interstitial collagen (%)</td>
<td>4.1±0.5</td>
<td>4.2±0.5</td>
<td>3.8±0.4</td>
<td>5.3±0.7</td>
<td>0.046</td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D. LV = left ventricle; RV = right ventricle; MCA = myocyte cross-sectional area. Diet (factor vitamin D content in the diet) = p-value of the vitamin D content in the diet effect; Time (factor length of the nutrient restriction) = p-value of the time effect; Diet x time = p-value of the interaction. When interactions were observed, the same superscript letters show the significant differences (⁴C4 ≠ D4; ²C2≠C4; ³D2 ≠ D4).
Table 5. Echocardiography data

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>p-value</th>
<th>Diet</th>
<th>Time</th>
<th>Diet x Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C2</td>
<td>D2</td>
<td>C4</td>
<td>D4</td>
<td></td>
</tr>
<tr>
<td>LVDD/tibia</td>
<td>1.60±0.02</td>
<td>1.63±0.02</td>
<td>1.63±0.02</td>
<td>1.72±0.02</td>
<td><strong>0.006</strong></td>
</tr>
<tr>
<td>LVWT (mm)</td>
<td>1.32±0.04</td>
<td>1.33±0.04</td>
<td>1.35±0.04</td>
<td>1.47±0.04</td>
<td>0.083</td>
</tr>
<tr>
<td>RWT</td>
<td>0.42±0.01</td>
<td>0.42±0.01</td>
<td>0.40±0.01</td>
<td>0.41±0.01</td>
<td>0.745</td>
</tr>
<tr>
<td>LA/tibia</td>
<td>0.09±0.002</td>
<td>0.09±0.002</td>
<td>0.08±0.002</td>
<td>0.10±0.002</td>
<td>&lt;<strong>0.001</strong></td>
</tr>
<tr>
<td>RAA (mg/dl)</td>
<td>0.17±0.01</td>
<td>0.16±0.01d</td>
<td>0.18±0.01b</td>
<td>0.20±0.01b</td>
<td>0.381</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>499±26.5c</td>
<td>520±27.1d</td>
<td>585±27.9b,c</td>
<td>722±26.5b,d</td>
<td>0.004</td>
</tr>
<tr>
<td>E (cm/s)</td>
<td>76.8±4.1</td>
<td>79.1±4.3</td>
<td>84.3±4.4</td>
<td>76.4±4.4</td>
<td>0.498</td>
</tr>
<tr>
<td>A (cm/s)</td>
<td>62.4±4.1</td>
<td>65.4±4.2</td>
<td>61.9±4.3</td>
<td>67.2±4.1</td>
<td>0.322</td>
</tr>
<tr>
<td>E/A</td>
<td>1.3±0.10</td>
<td>1.27±0.10</td>
<td>1.46±0.11</td>
<td>1.26±0.10</td>
<td>0.209</td>
</tr>
<tr>
<td>TRIV (ms)</td>
<td>21.8±1.2</td>
<td>22.6±1.2</td>
<td>21.1±1.6</td>
<td>20.5±1.2</td>
<td>0.935</td>
</tr>
<tr>
<td>LVSD/tibia</td>
<td>0.56±0.03</td>
<td>0.62±0.03</td>
<td>0.61±0.03</td>
<td>0.73±0.03</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>FS</td>
<td>0.65±0.02</td>
<td>0.62±0.02</td>
<td>0.62±0.02</td>
<td>0.58±0.02</td>
<td><strong>0.018</strong></td>
</tr>
<tr>
<td>EF</td>
<td>0.95±0.01</td>
<td>0.94±0.01</td>
<td>0.94±0.01</td>
<td>0.92±0.01</td>
<td><strong>0.023</strong></td>
</tr>
</tbody>
</table>

The data are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D. LVDD = left ventricular (LV) end-diastolic diameter; LVWT = LV wall thickness; RWT = relative wall thickness (2x LVWT/LVDD); LA = left atrium; RAA = right atrium area; LVM = left ventricle mass; E = E wave; A = A wave; TRIV = isovolumetric relaxation time; LVSD = LV end-systolic diameter; FS = fractional shortening; EF = ejection fraction. Diet (factor vitamin D content in the diet) = p-value of the vitamin D content in the diet effect; Time (factor length of the nutrient restriction) = p-value of the time effect; Diet x time = p-value of the interaction. When interactions were observed, the same superscript letters show the significant differences (aC2≠D2; bC4 ≠ D4; cC2≠C4; dD2 ≠ D4).
Figure Legends

Figure 1. Study design - C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D.

Figure 2. The data are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D; PTH = serum parathyroid hormone levels. Diet p-value factor < 0.001; Time p-value factor = 0.442; Diet x time p-value interaction = 0.856); Diet x time p-value interaction = 0.880).

Figure 3. The data are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D. 25(OH)D3 = serum vitamin D3 levels. Diet p-value factor < 0.001; Time p-value factor = 0.079; Diet x time p-value interaction = 0.018). Interactions were observed and the significant differences are shown.

Figure 4. The data are expressed as the mean ± the standard error of the mean. C2 = 2 months on a diet with 1000 IU/kg of vitamin D; D2 = 2 months on a diet with no vitamin D; C4 = 4 months on a diet with 1000 IU/kg of vitamin D; D4 = 4 months on a diet with no vitamin D; Panel A - OHADH = beta-hydroxyacyl coenzyme-A dehydrogenase. Diet p-value factor < 0.001; Time p-value factor = 0.442; Diet x time p-value interaction < 0.001. Interactions were observed and the significant differences are shown. Panel B - LDH = lactate dehydrogenase. Diet p-value factor < 0.001; Time p-value factor < 0.001); Diet x time p-value interaction < 0.001. Interactions were observed and the significant differences are shown.
Impact of the Length of Vitamin D Deficiency on Cardiac Remodeling

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