Ventricular Remodeling Induced by Tissue Vitamin A Deficiency in Rats

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Key Words

Inflammation • Oxidative stress • Energetic metabolism
• Vitamin A • Remodeling

Abstract

Background/Aims: Experimental studies suggest that vitamin A plays a role in regulating cardiac structure and function. We tested the hypothesis that cardiac vitamin A deficiency is associated with adverse myocardial remodeling in young adult rats. **Methods:** Two groups of young female rats, control (C - n = 29) and tissue vitamin A deficient (RVA - n = 31), were subjected to transthoracic echocardiography exam, isolated rat heart study and biochemical study. **Results:** The RVA rats showed a reduced total vitamin A concentration in both the liver and heart [vitamin A in heart, $\mu\text{mol/kg}$ (C = 0.95 ± 0.44 and RVA = 0.24 ± 0.16 , $p = 0.01$)] with the same serum retinol levels (C = $0.73 \pm 0.29 \mu\text{mol/L}$ e RVA = $0.62 \pm 0.17 \mu\text{mol/L}$, $p = 0.34$). The RVA rats showed higher left ventricular diameters and reduced systolic function. The RVA rats also demonstrated increased lipid hydroperoxide/total antioxidant capacity ratio and

cardiac levels of IFN- γ and TNF- α but not of metalloproteinase (MMP)-2 and -9 activity. On the other hand, the RVA rats had decreased levels of β -hydroxyacylcoenzyme A dehydrogenase and lactate dehydrogenase. **Conclusions:** Tissue vitamin A deficiency stimulated cardiac remodeling and ventricular dysfunction. Additionally, the data support the involvement of oxidative stress, energy metabolism, and cytokine production in this remodeling process.

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Introduction

Vitamin A is essential for several physiological processes, especially those related to cell differentiation and growth. Experimental studies suggest that retinoic acid (RA), one of the compounds representing vitamin A biological activity, plays a role in regulating cardiac structure and function throughout life [1]. During the early stages of cardiogenesis, excess RA produces congenital defects related to abnormal folding and septation of the

outflow tract and cardiac chambers [2]. In the adult rat, an excess of RA signaling induced by overexpression of the RA receptors or retinoid X receptors, results in cardiomyocyte abnormalities and dilated cardiomyopathy [3]. The supplementation of physiological RA provokes a physiological hypertrophy with a normal interstitial collagen matrix, a proportional increase in cardiac mass and volume, and improved cardiac function [1, 4]. In contrast, vitamin A-deficient animals and receptor knockout models have thin myocardial walls, and the embryos develop a generalized edema associated with heart failure [5].

Considering the effects of vitamin A following different cardiac injuries, several experimental studies have shown that RA suppresses the morphological changes of the heart and alterations in gene expression associated with hypertrophy induced by angiotensin II and endothelin [6, 7], and it attenuates ventricular remodeling associated with hypertension [8] after myocardial infarction [9] and in aortic-banded rats [6].

Vitamin A is transported mainly to the heart bound to retinol-binding protein (RBP). This complex (retinol-RBP) is maintained at nearly constant levels over a wide range of liver retinol concentrations. After meals containing vitamin A, retinyl esters, and, to a lesser extent, retinol are present in the circulation in chylomicrons, chylomicron remnants, and other lipoproteins [10]. The majority of these chylomicrons are cleared from the circulation by the liver. However, significant percentages (25-30%) of chylomicrons rich in retinoid are cleared from the circulation by extrahepatic tissues [11]. It is accepted that as long as plasma retinol remains at or above an accepted level, target tissues that use vitamin A will receive an adequate supply of retinol from the circulation.

Recently, Ross & Li [10] showed that an adequate concentration of plasma retinol alone is not sufficient to maintain tissue retinol levels under circumstances in which dietary vitamin A intake is low or absent. However, it is not known whether cardiac vitamin A deficiency has any cardiac effect in young adult animals. Accordingly, the following study was designed to test the hypothesis that tissue vitamin A deficiency in young adult female rats is associated with adverse myocardial remodeling. In addition, it is accepted that remodeling is modulated by several pathophysiological events, including alterations in the extracellular matrix, increased oxidative stress, inflammatory cytokines, and shifts in the cardiac energy metabolism. Therefore, the adverse consequences induced by tissue vitamin A deficiency were assessed by echocardiography, hypertrophy level, isolated hearts,

cardiac oxidative stress, inflammation markers, energy metabolism variables, collagen amounts, and metalloproteinase activity.

Materials and Methods

All experiments and procedures were performed in accordance with NIH guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of our institution.

Study design

Our protocol was adapted from Gardner and Ross [12]. Considering that tissue vitamin A concentration is high in the normal situation, this protocol reduces the transfer of vitamin A in milk to the nursing pups and shortens the onset of local vitamin A deficiency. Female Wistar rats were utilized. Control animals (C) were born to dams fed an AIN-93 diet sufficient in vitamin A (4 retinol equivalents (RE)/g diet) during pregnancy and lactation. It is recommended to use animals of the same sex, as this variable can be an important regulation factor of the cardiac remodeling in response to several stimuli. Post weaning, the animals were fed the same diet. Dietary vitamin A-deficient rats (RVA) were born to dams fed an AIN-93 vitamin A-free diet during pregnancy and lactation. After weaning, rats were fed AIN-93 containing 0.18 RE/g diet. These dams were fed a modified AIN-93G diet devoid of vitamin A after arrival to reduce the transfer of vitamin A in milk to the nursing pups [13]. To show that animals at birth had normal vitamin A status, a group of pups were euthanized to measure the vitamin A levels in the livers and hearts. After weaning, the food intake and the body weight of the rats were measured weekly. No differences were observed between the groups. At the age of approximately 70 days, young adult female rats (C - n=29; RVA - n=31) were submitted to transthoracic echocardiography exam, *in vitro* functional heart study, and measurements of vitamin A (serum, liver and heart), ventricular hypertrophy, fibrosis, hepatic and cardiac oxidative stress, inflammation markers, energetic metabolism, and metalloproteinase activity.

Echocardiography study

A group of 12 control animals and 15 RVA animals were weighed and evaluated by transthoracic echocardiography exam, as previously described [4].

In vitro left ventricular function

Animals were anesthetized with thiopental sodium (50 mg/kg, IP) and heparinized (2000 UI, IP). Ventricular pressure was measured by using a 23XL transducer and a Gould as previously published [14].

Morphometric analysis

The right and left ventricles (including the interventricular septum) were dissected, separated, and weighed. Myocyte cross-sectional area and interstitial collagen volume fraction were determined as described previously [4].

Fig. 1. Liver and heart total vitamin A concentration measured in newborn pups. (A) Serum, liver and heart total vitamin A concentration in young adult female rats. (B) Values are mean $\pm$ SD; RVA: vitamin A-restricted group.

Vitamin A analysis

Serum retinol was extracted by using the modified extraction method reported by Folch et al. [15]. Total vitamin A in cardiac and liver tissue was also assayed by an HPLC system after saponification as previously described [16]. Six to nine animals from each group were used to assay vitamin A status. In the pups, liver and heart total vitamin A levels were determined among four pooled samples ($n=6$ neonates/treatment in each pool) after saponification, extraction, and reverse-phase HPLC. All-trans retinoic acid was extracted by using the method described in Kane MA et al. [17]. The cardiac all-trans RA levels were assayed by HPLC system using the method reported by Schmidt CK et al., excepted that the column used was Supersil ODS RP, 4,6X150 mm, 3 μ [18].

Myocardial and hepatic oxidative stress

Lipid hydroperoxide, total antioxidant status, glutathione peroxidase (GSHPx), superoxide dismutase (SOD), and catalase (CAT) were assessed as previously specified. The spectrophotometric determinations were performed as previously published [19].

Metalloproteinase-2 and -9 activity

The activity of metalloproteinase (MMP)-2 and -9 was adapted from Tyagi et al. [20]. In brief, the gel was incubated at 37°C overnight in activation solution consisting of 50 mM Tris pH 8.4 and 5 mM CaCl_2 . The staining and destaining were performed as previously described [21]. The gels were photographed, and the intensity of gelatinolytic action (clear bands) was analyzed in a UVP, UV, White Darkhon image analyzer.

	C N=12	RVA N=15	P
LVDD (mm)	5.8 $\pm$ 0.43	6.2 $\pm$ 0.37	0.042
LVDS (mm)	1.9 $\pm$ 0.51	2.4 $\pm$ 0.32	0.008
DTPW/LVDD	0.22 $\pm$ 0.03	0.20 $\pm$ 0.02	0.060
LAD/AD	0.97 $\pm$ 0.12	1.10 $\pm$ 0.17	0.021
FS (%)	66.2 $\pm$ 7.50	60.2 $\pm$ 4.72	0.019
EF (%)	96.0 $\pm$ 3.0	93.0 $\pm$ 2.0	0.049
E/A	1.46 $\pm$ 0.34	1.41 $\pm$ 0.27	0.726

Table 1. Echocardiographic data. C: control group; RVA: vitamin A restriction group; LVDD: left ventricular diameter at diastole; LVDS: left ventricular diameter at systole; DTPW: diastolic thickness of posterior wall; LAD: left atrial diameter; AD: aortic diameter; FS: fractional shortening; EF: ejection fraction; E/A: ratio between the E and A-waves evaluated in transmitral flow. For normally distributed variables, values are means $\pm$ SD and were compared through Student's *t* test. For non-normally distributed variables, values are medians (lower quartile - upper quartile) and were compared through the Mann-Whitney test.

Energy metabolism

LV samples of 200 mg were weighted and homogenized. The supernatant was analyzed for total protein, lactate dehydrogenase (LDH; E.C.1.1.1.27.), β -hydroxyacylcoenzyme

	C N=9	RVA N=11	p
LVP (mmHg)	181 ± 24.9	175 ± 20.6	0.561
+dp/dt max (mmHg/s)	5513 ± 1394	4229 ± 873	0.027
-dp/dt max (mmHg/s)	2800 ± 324	2708 ± 359	0.553

Table 2. Isovolumetrically beating isolated heart study. C: control group; RVA: vitamin A restriction group; LVP: peak of isovolumetric pressure; +dp/dt: maximum rate of ventricular pressure rise; -dp/dt: decreased maximum rate of ventricular pressure rise. Values are means ± SD and were compared through Student's *t* test.

	C N=5	RVA N=5	P
LH (nmol/g)	164 (161-169)	211 (189-246)	0.032
TAS (%)	36.8 (36.3-37.7)	31.4 (28.1-34)	0.056
LH/TAS (nmol/g)	4.40 (4.30-4.60)	6.70 (5.60-8.80)	0.032
Cat (μmol/g)	167 ± 35.0	161 ± 27.0	0.794
GSH-Px (nmol/mg)	35.6 ± 5.01	40.5 ± 21.6	0.630
SOD (nmol/mg)	1.40 ± 0.03	1.38 ± 0.02	0.610
TP (%)	14.4 (13.9-15.5)	14.8 (14.7-16.9)	0.220

Table 3. Cardiac determination of oxidative stress. C: control group, RVA: vitamin A restriction group; LH: lipid hydroperoxide; TAS: total antioxidant substances, Cat: catalase; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; TP: total protein. For normally distributed variables, values are mean ± SD and were compared through Student's *t* test. (For non-normally distributed variables, values are medians (lower quartile - upper quartile) and were compared through the Mann-Whitney test).

A dehydrogenase (OHADH; E.C.1.1.1.35.), and citrate synthase (CS; E.C.4.1.3.7.) as previously published [22]. The spectrophotometric determinations were performed as previously specified [19].

Evaluation of cytokine production

Briefly, 60 mg of cardiac tissue was homogenized and solubilized in 50 mM potassium phosphate buffer, pH 7.4; 0.3 M sucrose; 0.5 mM DTT; 1 mM EDTA, pH 8.0; 0.3 mM PMSF; 10 mM NaF; and 1:100 protease inhibitor. Cytokine levels in the cardiac homogenate were evaluated by ELISA according to manufacturer instructions (R & D Systems, Minneapolis, MN, USA). The sensitivity of the ELISA for IFN- γ and TNF- α were 19 and 31 pg/mL, respectively.

Statistical methods

Comparisons between groups were made by Student's *t* test for parameters with normal distribution. Otherwise, groups were compared by the Mann-Whitney U test. The data were expressed as the mean ± SD or medians (including the lower and upper quartiles) and analyzed with SigmaStat for Windows v2.03 (SPSS Inc, Chicago, IL). The significance level was 5%.

Results

The pups whose mothers were fed the vitamin A-restricted diet showed no alterations in the levels of total vitamin A in the heart (C = 0.82 ± 0.37 μmol/kg and RVA = 0.90 ± 0.35 μmol/kg, *p* = 0.8) or liver (C = 23.3 ± 11.7 μmol/kg and RVA = 24.3 ± 8.7 μmol/kg, *p* = 0.91) when compared to control animals. The RVA rats showed reduced total vitamin A concentrations in both the liver (C = 1007 ± 249 μmol/kg and RVA = 125 ± 50 μmol/kg, *p* < 0.001) and the heart (C = 0.95 ± 0.44 μmol/kg and RVA = 0.24 ± 0.16 μmol/kg, *p* = 0.01) with the same serum retinol levels (C = 0.73 ± 0.29 μmol/L e RVA = 0.62 ± 0.17 μmol/L, *p* = 0.34) (Fig. 1). Likewise, RVA rats showed reduced retinoic acid concentration in the heart (C = 0.179 ± 0.056 μmol/kg of tissue and RVA = 0.074 ± 0.030 μmol/kg of tissue, *p* = 0.006).

There was no difference in the heart rate between the two groups (C = 335 ± 58 and RVA = 321 ± 44 , *p* = 0.470). Echocardiographic findings are shown in

	C	RVA	P
LH (nmol/g)	357 ± 50.9	358 ± 19.6	0.953
TAS (%)	21.4 ± 2.18	21.2 ± 1.03	0.861
LH/TAS (nmol/g)	17.0 ± 4.6	16.9 ± 1.7	0.971
GSH-Px (nmol/mg)	105 (100.6-117.3)	107 (104.6-112.7)	0.670
SOD (nmol/mg)	2.6 ± 0.1	2.4 ± 0.1	0.019

Table 4. Hepatic determination of oxidative stress. C: control group; RVA: vitamin A restriction group; LH: lipid hydroperoxide; TAS: total antioxidant substances, Cat: catalase; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase; TP: total protein. For normally distributed variables, values are mean ± SD and were compared through Student's *t* test. (For non-normally distributed variables, values are medians (lower quartile - upper quartile) and were compared through the Mann-Whitney test).

	C N=5	RVA N=5	P
MMP-2	7.59 ± 0.7	7.47 ± 0.4	0.735
MMP-9	2.02 ± 0.7	2.40 ± 0.9	0.464
TNF-α (pg/ml)	188.1 ± 33.9	294.4 ± 80.5	0.026
IFN-γ (pg/ml)	156.4 ± 31.7	257.9 ± 70.2	0.019
β-OH (nmol/mg/protein)	52.1 ± 9.4	42.0 ± 14.7	0.06
LD (nmol/mg/protein)	88.9 ± 11.1	78.9 ± 11.3	0.042
CS (nmol/mg/protein)	346 ± 85	349 ± 54	0.934

Table 5. Cardiac biochemical data. C: control group; RVA: vitamin A restriction group; MMP-2: metalloproteinase-2 activity/latent ratio; MMP-9: metalloproteinase-9 activity/latent ratio; IFN-γ: interferon-γ; TNF-α: tumor necrosis factor-α; β-OH: β-hydroxyacylcoenzyme A dehydrogenase; LD: lactate dehydrogenase; CS: citrate synthase. For normally distributed variables, values are means ± SD and were compared through Student's *t* test. (For non-normally distributed variables, values are medians (lower quartile - upper quartile) and were compared through the Mann-Whitney test).

Table 1. The vitamin A-restricted group showed higher end-diastolic left ventricular diameter, systolic left ventricular diameters and a left atrium/aortic diameter ratio compared to the control group, suggesting dilatation of the left heart. Ejection fraction and fractional shortening ($C = 66.2 \pm 7.50\%$ and $RVA = 60.2 \pm 4.72\%$, $p = 0.019$) were lower in the vitamin A-restricted group, suggesting systolic dysfunction.

Considering morphometric variables, there was no differences between the groups in the LV weight/body weight ratio ($C = 2.51$ g/mg (2.42-2.67) and $RVA = 2.49$ g/mg (2.40-2.60), $p = 0.704$), myocyte cross-sectional area ($C = 237 \pm 18$ μm^2 and $RVA = 230 \pm 13$ μm^2 , $p = 0.314$), and interstitial collagen volume fraction ($C = 2.78 \pm 1.10\%$ and $RVA = 3.50 \pm 1.57$, $p = 0.343$), suggesting that there

is no hypertrophy or fibrosis in the vitamin A-restricted group.

Table 2 shows the functional data for rats submitted to the isovolumetrically beating isolated heart procedure. The maximum rate of ventricular pressure rise (+dp/dt) was lower in the RVA group ($C = 5513 \pm 1394$ mmHg/s and $RVA = 4229 \pm 873$ mmHg/s, $p = 0.027$) than in the control group, suggesting systolic dysfunction in the vitamin A-restricted group.

Tables 3 and 4 display the lipid hydroperoxide findings in the heart and liver. The heart lipid hydroperoxide level ($C = 164$ nmol/g (161-169) and $RVA = 211$ nmol/g (189-246), $p = 0.032$) and ratio between heart lipid hydroperoxide and total antioxidant substances ($C = 4.4$ nmol/g (4.3-4.6) and $RVA = 6.70$ nmol/g

(5.60-8.80), $p = 0.032$) were higher in the vitamin A-restricted group, suggesting increased oxidative stress. Heart antioxidant enzyme systems were not affected by vitamin A restriction. Liver lipid hydroperoxide was similar in the two groups, but superoxide dismutase was consumed in the RVA group.

Table 5 shows the biochemical data. Considering MMP-2 and -9 activity, there were no differences between the groups. In relation to energy metabolism, the vitamin A-restricted group showed decreased levels of β -hydroxyacylcoenzyme A dehydrogenase at the limit of statistical significance ($C = 52.1 \pm 9.4$ nmol/mg protein and $RVA = 42.0 \pm 14.7$ nmol/mg protein, $p = 0.06$) and of lactate dehydrogenase ($C = 88.9 \pm 11.1$ nmol/mg protein and $RVA = 78.9 \pm 11.3$ nmol/mg protein, $p = 0.042$), suggesting decreased energy substrate. There was no difference in the citrate synthase levels. Considering the cytokine production, the vitamin A-restricted group showed increased cardiac levels of IFN- γ ($C = 156.4 \pm 31.7$ pg/mg of protein, $RVA = 257.9 \pm 70.2$; $p = 0.019$) and TNF- α ($C = 188.1 \pm 33.9$ pg/mg of protein, $RVA = 294.4 \pm 80.5$ pg/mg of protein; $p = 0.026$), suggesting increased cardiac inflammation.

Discussion

The main finding of this study is that even when serum retinol is similar to the control levels and liver storage was not depleted, vitamin A-requiring target organs, such as the heart, may still receive insufficient retinol. Furthermore, differences in heart retinol levels are associated with physiological differences. The tissue vitamin A deficiency induced ventricular remodeling characterized by increased left chamber size and ventricular dysfunction.

It has been shown that vitamin A deficiency induced in the embryonic period is associated with several cardiac alterations [1, 22]. However, because our experiment showed that the total vitamin A concentration in the liver and heart were the same in the comparison of newborns of both groups - adequate and restricted diet - it is likely that the cardiac findings are not associated with vitamin A deficiency in the embryonic period.

The study protocol did not induce systemic vitamin A deficiency in the young adult female rats. We found that the restricted group had serum retinol values similar to those of the control group. As far as the vitamin A liver contents, they were lower in the restricted group, but this cannot be considered as indicating vitamin depletion [12].

Interestingly, the vitamin A heart content in the restricted group was diminished, a finding that indicates the importance of the dietary vitamin A to provide enough retinol to vitamin A-requiring target tissues. This phenomenon was also described in the lungs by Ross and Li [10].

Another important finding of our study was that tissue vitamin A deficiency induced ventricular enlargement, as revealed by increases in the left ventricular end-diastolic dimension. It has been well established that ventricular dilation plays a key role in the ventricular remodeling process [23, 24]. Considering that there was no evidence of hypertrophy in the tissue vitamin A deficiency group, this phenomenon could be a consequence of myocyte slippage. Additionally, it was observed that enlargement of this chamber was associated with systolic dysfunction. The echocardiographic data shows that systolic function parameters, such as ejection fraction and fractional shortening, were lower in the RVA group, while end-systolic volume was higher in the RVA group. Furthermore, myocardial contractility, evaluated by isovolumetric beating isolated heart, may be assessed by the maximum rate of ventricular pressure rise ($+dp/dt$), which was lower in the RVA group. One of the most striking characteristics of cardiac remodeling is that it invariably results in a progressive decrease in ventricular function. Biochemical, genetic, and structural alterations can be responsible for progressive ventricular dysfunction [23, 24].

Considering the pathophysiological mechanisms involved in the remodeling process, it is universally accepted that extracellular matrix is important to normal ventricular geometry and function. Loss of collagen fibrils and struts induced by MMP are associated with myocyte slippage and impaired contraction. Increased MMP activity in rats with acute myocardial infarction, for instance, was associated with progressive ventricular dilation. In addition, pharmacological inhibition of MMP in AMI animals attenuated the remodeling process, with resulting functional preservation [25]. Likewise, collagen accumulation is associated with conduction abnormalities and progressive cardiac dysfunction [26]. However, in the present study, the observation of similar values of MMP-2 and -9 and interstitial collagen volume fraction between the groups suggests that these variables are not involved in the cardiac remodeling induced by tissue vitamin A deficiency.

It is well established that reactive oxygen species and oxidative stress play a critical role in cardiac remodeling following several cardiac injuries. To protect

against oxidative stress, a well-organized system of antioxidants works in a coordinated manner and is separated into both enzymatic (superoxide dismutase, glutathione peroxidase, and catalase) and non-enzymatic antioxidants (GSH, uric acid, coenzyme Q10, etc.). Importantly, there is an association between vitamin A and oxidative stress [27]. For instance, Choudhary et al. [28] showed that retinoic acid prevented angiotensin II and stretch-induced apoptosis, by inhibiting ROS generation and increasing the anti-oxidant defense system. Additionally, it has been shown that vitamin A deficiency increases heart oxidative stress [29]. In the present study, there was an increase in oxidative stress in hearts with reduced concentrations of vitamin A. In addition, the enzymatic antioxidants were not different in comparison with control rats, suggesting the participation of the non-enzymatic antioxidant system. Therefore, our data suggest the involvement of oxidative stress in cardiac remodeling induced by tissue vitamin A deficiency.

Considering the role of cytokines, in several heart failure models, cytokine production can modulate left ventricular remodeling. Indeed increased IFN- γ , and mainly TNF- α levels are associated with left ventricular dysfunction, abnormalities in myocardial energetic metabolism, cachexia, activation of fetal gene programs, apoptosis, hypertrophy, and fibrosis [30]. Importantly, in this model, the remodeling intensification was also

associated with altered IFN- γ and TNF- α levels. Therefore, at this point, our data suggest that IFN- γ and TNF- α stimulation might be involved in this remodeling process.

Another important issue is that cardiac remodeling can be associated with abnormalities in myocardial energy metabolism. Under normal conditions, the oxidation of fatty acids covers more than 70% of the cardiac energy need. In different models, the morphological and functional alterations are related to an increased utilization of glucose as a substrate and a decreased in cardiac substrate utilization [31]. In our study, there was a decrease in both carbohydrate (assessed by lactate dehydrogenase) and fatty acid (assessed by β -hydroxyacylcoenzyme A dehydrogenase) oxidation. Our data are in accordance with a recent study that showed that lipid energy metabolism was significantly modified in hearts of vitamin A-deficient rats [32]. Therefore, these metabolic changes are thought to contribute to the development of cardiac remodeling induced by tissue vitamin A deficiency.

In conclusion, the reduced cardiac levels of vitamin A found in RVA hearts lead to a remodeling process, evidenced by enlargement of the left cardiac chamber and systolic dysfunction associated with increased oxidative stress, abnormalities in energy metabolism, and increased IFN- γ and TNF- α levels.

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