Model of Long-Term Vitamin A Deficiency in the Mammary Gland of Virgin Rats

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Abstract

Deficiency in vitamin A is an evil in underdeveloped countries where the minimum recommended intake is not achieved. This may have long-term implications on the health of the population. For this reason, it would be very useful to achieve an animal model where the different implications of this deficiency can be observed. This could lead to the formulation of future nutrition politics. The levels of retinoic acid and intermediary of the vitamin A pathway were studied; The levels of retinoic acid and intermediary of the vitamin A pathway (CRBP-1 and RARα) were studied. There was a decrease in the values of retinoic acid main indicator of deficiency of this vitamin in serum, liver and mammary gland and variation in expression of CRBP-1 and RARα mRNA in mammary gland. This model provides a tool for the study of the metabolism of vitamin A and its effects in different organs and especially in virgin mammary gland, where a deficiency is achieved despite being in a state of latency.

Subject Areas

Nutrition

Keywords

Retinoic Acid, Nutrition, CRBP-1, RARα

1. Introduction

Worldwide, vitamin A deficiency (VAD) affects an estimated of 190 million preschool-aged children and 19.1 million pregnant women [1]. It has been estimated that 44.4% of pre-school children in Africa would be at risk of VAD [2]. In Ethiopia, this deficiency leads to 80,000 deaths per year and affects 61% of

pre-school children [3]. Vitamin A deficiency remains a widespread public health problem among women and children in the developing world [4], and it increases morbidity and mortality due to increased susceptibility to infection [5].

Vitamin A and its derivatives (referred to as retinoids) are essential dietary compounds and are key regulators of cell differentiation, proliferation, and death. It is estimated that more than 500 genes are regulated by retinoic acid [6], through the binding and activation of the different isoforms of retinoic acid receptors (RARα, β and γ) and retinoid X receptors (RXR), which are members of the nuclear receptor family. Adult animals deprived of vitamin A display severe abnormalities including dysfunction of epithelia of mammary gland [7] [8].

The nutritional requirements of vitamin A have been calculated through studies in which attempts have been made to correct experimentally produced deficiency states. Current recommendations from the Food and Nutrition Board of the National Research Council are based on the amount of retinoids needed to cover variations between absorption and utilization. VAD is recognized as a nutritional problem in many countries. VAD is considered to affect growth only under severe deficit conditions of (0.7 µmol/l) [9] [10] [11] [12]. In fasting circulation, retinol (the predominant vitamin A species) bound to RBP (Retinol Binding Protein) is found at a normal concentration ranging from 2 to 4 µM in humans and about 1 µM in rodents [13].

The study of the effects of marginal vitamin A deficiency is of great importance as it reflects situation of many human beings, particularly those in developing countries. For this reason, an animal model of deficiency should be used to study the metabolic changes.

2. Materials and Methods

Animals and Diets

Wistar rats are a good model for nutritional studies. Female Wistar rats, bred in our animal facilities (IMIBIO, National University of San Luis, Argentina), were weaned at 21 days old and immediately randomly assigned to either the experimental group (standard diet, devoid of vitamin A [VAD group]), the control (CO) group (standard diet with 4000 IU of vitamin A [8 mg retinol as retinyl palmitate] per kilogram of diet) or refed group (REF). The experimental period was 6 months for VAD and CO and 5 months with VAD diet and 30 days with control diet for the REF group. REF group was used to study the reversibility of the possible changes caused by the vitamin deficiency. Diets, mineral mix and vitamin mix were prepared according to the AIN-93 for laboratory rodents [14]. The composition (grams per kilogram diet) of experimental and CO diets are shown in Tables 1-3. The rats were kept in a 21°C - 23°C controlled environment with a 12-hour light:dark cycle. They were given free access to food and water throughout the entire experimental period. After the entire treatment period, 4 rats from each group (CO, VAD and REF) were euthanized by CO_2 inhalation. The blood was collected without anticoagulant in order to obtain the se-

rum. For this purpose, the samples were washed with H_2O at 37˚C for 20 min. The serum was then centrifuged 2 times at 3000 rpm for 10 min. Then the inguinal mammary gland and liver were separated. The tissue fractions were maintained at −70˚C. The samples for the determination of retinol were separated and protected from light, in order to decrease the photoisomerization of vitamin A. We followed the general guidelines for the care and use of laboratory animals recommended by the Animal Care Committee of the National University of San Luis.

3. Retinol Concentration Analyses

The retinol concentration was determined by the modified technique Neeld and

Table 1. Ingredient composition of the diet fed to rats.

Ingredients	g/kg diet
Corn starch	397.5
Sucrose	100
Dextrinized corn starch	132
Lactalbumin	200
Soybean oil	70
Cellulose fiber	50
AIN-93 mineral mix	35
AIN-93 vitamin mix	10
L-cystine	3
Choline bitartrate	2.5
Tert-butylhydroquinone	0.0014

Table 2. AIN-93 vitamin mix.

Vitamin	g/kg diet (CO)	g/kg diet (VAD)
Nicotinic acid	3.000	3.000
Calcium Pantothenate	1.600	1.600
Pyridoxine–HCl	0.700	0.700
Thiamine-HCl	0.600	0.600
Riboflavin	0.600	0.600
Folic acid	0.200	0.200
D- Biotin	0.020	0.020
Vitamin B-12 (cianocobalamina)	2.500	2.500
Vitamin E (500 UI/g)	15.000	15.000
Vitamin A (*trans*-retinilpalmitato)	**0.800**	-
Vitamin D3 (400.000 UI/g)	0.250	0.250
Vitamin K	0.075	0.075
Sucrose	974.655	975.455

Table 3. AIN-93 mineral mix.

Minerals	mg/kg diet
a) **Essential mineral elements:**	
Calcium carbonate, anhydrous	357.00
Potassium phosphate, monobasic	196.00
Potassium citrate, monohydrate	70.78
Sodium chloride	74.00
Potassium sulfate	46.60
Magnesium oxide	24.00
Ferric citrate	6.06
Zinc carbonate	1.65
Manganese carbonate	0.63
Cupric carbonate	0.30
Potassium iodide	0.01
Sodium selenate, anhydrous	0.01025
Ammonium Paramolybdate. $4H_2O$	0.00795
b) **Potentially beneficial elements:**	
Sodium metasilicate. $9H_2O$	1.4500
Chromium and potassium sulphate. $12H_2O$	0.2750
Lithium chloride	0.0174
Boric acid	0.0815
Sodium Fluoride	0.0635
Nickel carbonate	0.0318
Ammonium Vanadate	0.0066
Sucrose	221.0260

Pearson [15]. The homogenates of liver or mammary gland and serum, was treated with 1 ml of 95% ethanol to precipitate proteins, and 1.5 ml of petroleum ether to extract vitamin A and carotenoids. It was centrifuged at 3000 rpm for 10 minutes at 37°C. The supernatant was read at 450 nm, corresponding to the absorbance of carotenes. Then was dried in an oven at 37°C and the residue was taken up in 50 μl of chloroform, 50 μl of acetic anhydride and 500 μl of TFA (Trifluoroacetic) is then added with vigorous stirring to 620 nm absorbance (OD620) and read within 30 seconds. In parallel a standard curve of vitamin A and carotenes process was made. Because the β-carotene reacts with TFA, the results were corrected after reading the absorbance at 450 nm and calculate the corresponding correction factor. All measurements were performed in duplicate.

4. RNA Isolation and RT-PCR Analysis

Total RNA was isolated from 150/200 mg of mammary tissue using the guanidi-

nium isothiocyanate-acid phenol method as modified by Puissant and Houdebine [16]. Ten micrograms of total RNA were reverse transcribed (RT) at 37˚C using random hexamer primers and Moloney murine leukemia virus retrotranscriptase (Invitrogen-Life Technologies, Buenos Aires, Argentina) in a 20 μL reaction mixture. The RNA was first denatured at 70˚C for 5 min in the presence of 2.5 μg of random hexamer primers (Invitrogen). For the subsequent RT reaction the following mixture was added: RT buffer [50 mM TriseHCl (pH8.4), 75 mM KCl, 3 mM MgCl$_2$], 0.5 mM dNTPs, 5 mM DTT, 200 units M-MLV Reverse Transcriptase. The reaction was incubated at 37˚C for 50 min, and then the reaction was inactivated by heating at 70˚C during 15 min. The cDNA was stored at 20˚C. The mRNA levels of CRBP-1, RARα and S28 were estimated by RT-PCR using rat-specific primers and reaction conditions described in Table 4. The PCR reactions were performed using a Biorad Thermocycler in a final volume of 20 μL. The reaction mixture consisted of 2 μL of 10X PCR Buffer, 1 μL of 50 mM MgCl$_2$, 0.4 μL of 10 mM dNTP Mix (Invitrogen), 0.25 μL of 5 U/mL Taq DNA Polymerase (Invitrogen), 0.1 μL of each 2.5 mM primer (forward and reverse primers) and 11 μL of diluted cDNA. The PCR reactions were initiated with 5 min incubation at 95˚C, followed by 40 cycles of 95˚C for 60 s, 60 s at the annealing temperatures shown in Table 4 and 72˚C for 60 s. Each PCR run included a notemplate control and a sample without RT. All measurements were performed in duplicate. RNA samples were assayed for DNA contamination by performing the different PCR reactions without prior RT. Relative levels of mRNA were normalized to the S28 reference gene. The resultant products obtained after PCR were separated by electrophoresis on 2% agarose gel containing GelRed. The image was visualized and photographed under UV transillumination.

5. Statistical Analysis

Results were expressed as mean values with their standard device. Statistical comparisons were made transversely between different dietary groups. The statistical significance between groups was determined by one-way ANOVA and the differences between the individual means were analyzed using Tukey's post hoc test. Differences having P values lower than 0.05 were considered to be statistically significant. Data analysis was done using the Graphpad prism 5 software. The images were taken with digital camera and the images analyzed with Image J software.

Table 4. PCR primers and conditions.

Gene	Primer sense (5'- 3')	Primer Antisense (5' - 3')	Tm (˚C)	Amplicon size	Gene Bank Accessions
CRBP-1	ACGTGGCCTTGCGAAAAATC	TCATGCACTTGCGGTCATCT	58	174 bp	NM 012733.4
RAR α	CGCCTGTGAGGGCTGTAAG	ATGCCCACTTCGAAGCATTT	61	150 bp	NM_031528
S28	GTGAAAGCGGGGCCTCACGATCC	GTACTGAGCAGGATTACCATGGC	59	289 bp	NR 046239.1

6. Results

6.1. Body Weight

When analyzing the animal weights, a significant decrease was observed in the experimental groups with VAD diet, from week 8 on diet intake, regarding the respective CO group (data not shown). It is noteworthy that the animals belonging to REF group had a weight gain equivalent to the CO group (Table 5). No differences were observed in the daily intake between the different experimental lots.

6.2. Retinoic Acid Levels

The content of retinoic acid in serum, liver and mammary gland was measured (Table 6). The dietary restriction of vitamin A for 24 weeks caused a decrease in retinoic acid levels in serum, liver and mammary gland with respect to CO group; indicating a specific state of nutritional deficiency of vitamin A. This state was reversed in serum, in liver and mammary gland with feedback of 4 weeks.

6.3. Effect of VAD on CRBP-1 mRNA expression

The expression of CRBP-1 in mammary gland was determined. Figure 1 shows the expression of CRBP-1 in mammary gland in the different experimental groups, where a decrease in expression is observed both in the group with deprived diet of vitamin A (VAD group) and in those subsequently supplemented (REF group).

6.4. Effect of VAD on RARα mRNA Expression

Expression of RARα was determined. Figure 2 shows RARα expression in mammary gland in the different experimental groups, where a decrease in

Table 5. Weights of the different experimental groups.

	CO	VAD	REF
Initial weight (g)	60.75 ± 4.85	54.50 ± 6.45	52.75 ± 5.79
Final weight (g)	341.5 ± 2.88[c]	296.3 ±16.76[d]	318.5 ± 9.14[e]
Weight Gain (g)	280.8 ± 2.22[c]	241.8 ± 1.15[d]	264.5 ± 5.68[c]

Values are expressed as the mean ± SD (n = 8). Medias with a different letter are statistically significant (p < 0.05).

Table 6. Retinoic acid levels in serum, liver and mammary gland.

	CO	VAD	REF
Serum (µmol/l)	1.79 ± 0.32[b]	0.06 ± 0.027[d]	1.92 ± 0.27[b]
Liver (µmol/g)	1.71 ± 0.20[b]	0.08 ± 0.03[a]	1.51 ± 0.06[b]
Mammary gland (µmol/g)	0.95 ± 0.04[b]	0.11 ± 0.08[a]	0.36 ± 0.03[c]

Values are expressed as the mean ± SD (n = 4). Medias with a different letter are statistically significant (p < 0.05).
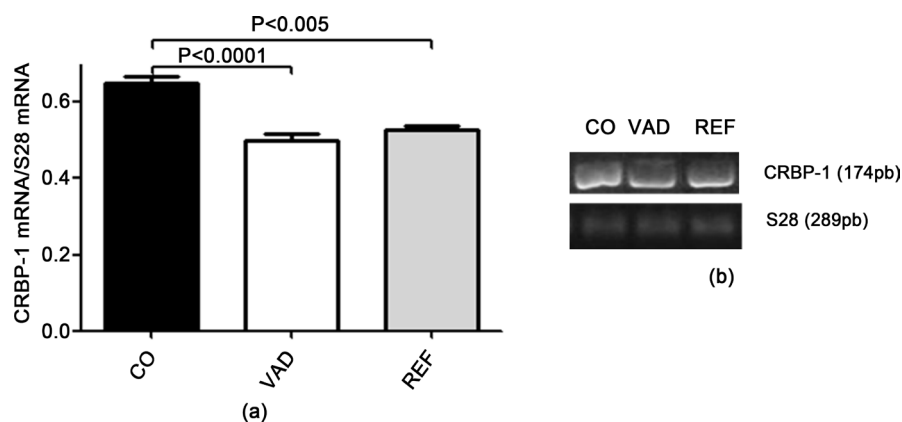
Figure 1. Expression of CRBP-1 in mammary gland by RT-PCR in the different experimental groups. (a) Semi-quantitative analysis of CRBP-1 expression. (b) Gel representative of the gene expression of CRBP-1 in the different experimental groups. Expression of CRBP-1 was performed using specific primers, and was adjusted for expression of the constitutive gene S28, as described in Materials and Methods. Representative image of agarose gel for CRBP-1 (174 pb) and S28 (284 bp) products.
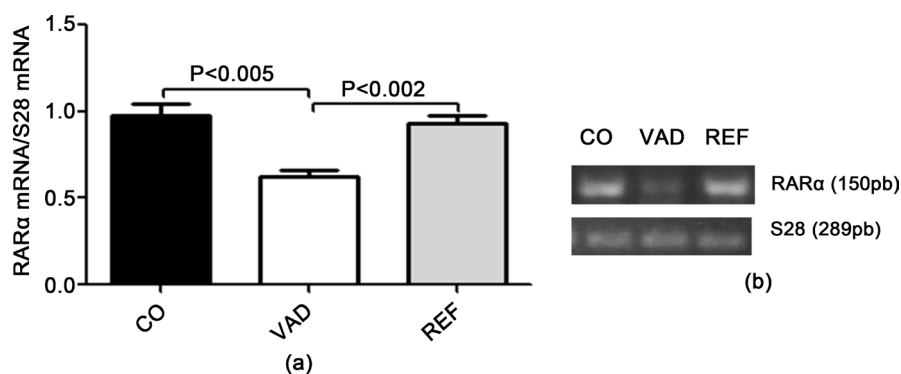

Figure 2. Expression of RARα in mammary gland by RT-PCR in the different experimental groups. (a) Semi-quantitative analysis of RARα expression. (b) Gel representative of the gene expression of RARα in the different experimental groups. Expression of RARα was performed using specific primers, and was adjusted for expression of the constitutive gene S28, as described in Materials and Methods. Representative image of agarose gel for RARα (150 pb) and S28 (284 bp) products.

expression in the VAD group and an increase after supplementation was observed. The 30-day diet with enough vitamin A diet was sufficient for the REF group to return to the control values.

7. Discussion

In the present study, a model of long-term deficiency is proposed for the study of the changes caused by the prolonged absence of vitamin A in virgin mammary gland.

In this model, a significant decrease in body weight gain was observed in VAD group. VAD is considered to affect growth only under conditions of severe deficit (<0.7 µmol/l) [9] [11] [12]. In rodents, the normal concentration of retinol is of the1 µM [13]. Vitamin A is an essential nutrient for mammalian growth and a

decrease in body weight gain and alterations in anthropometric measures has been observed in children with subclinical and clinical deficits [17].

In rodents, disturbances of vitamin A signaling, due to dietary depletion or genetic manipulation, may promote deregulation of adipose tissue [18]. Treatment of rodents with vitamin A or retinoic acid can change the expression levels of adipose genes involved in energy homeostasis [19].

In our model, dietary intake without vitamin A causes alterations in the amount of retinoic acid in serum, in liver (which is the reserve organ for vitamin A [20] [21], and in mammary gland. Our results demonstrate that levels of circulating retinoic acid decreased compared with the CO group. During the refed period, we could observe that the values of retinoic acid reached the values of the CO group. In liver and mammary gland of the VAD group, we observed that these levels were lower compared to the CO group. In liver, during the refed period, we could observe that the values of retinoic acid reached the values of the CO group. In mammary gland, the levels of retinoic acid observed increased with respect to the VAD group.

According to a study conducted by Ross [22], rats fed with a deprived diet of vitamin A showed retinoic acid deficiency in liver (<5 mg/g tissue) and plasma (<0.3 M) at 7 weeks age in males and 8 weeks of age in females. Moreover, external signs of vitamin A deficiency were manifested approximately one week later for both sexes [22]. These findings are consistent with our findings; where at 8 weeks in the VAD group the signs of Vitamin A deficiency were observed.

It has been shown that retinoic acid regulates cellular processes including cell proliferation, differentiation and apoptosis, and therefore plays important roles in embryo development and subsequent tissue maintenance [23] [24] [25]. Retinoic acid signaling is involved in the initiation of mammary gland development in the embryonic stage [26]. The RARs are the main mediators of the biological effects of vitamin A, with an established role in the maintenance of differentiated state of epithelial tissues [27]. In turn, the RAR signaling pathway has been shown to be defective in carcinomas of various organs, such as the mammary gland, mainly because of reduced expression of RARβ or CRBP-1 [28] [29]. However, if these alterations affect the oncogenesis or maintenance of the tumor, it still remains unresolved. It has been reported that nutritional status in vitamin A would alter the expression of the different RAR subtypes in tissues [30] [31] [32] [33]. In our model, a decrease in RARα expression was observed in the VAD group, whereas during the supplementation period, these levels reached similar values to the CO group. This situation would be suggesting that RARα levels would be related to vitamin A concentration.

From the model of null mice in CRBP-1 this protein is proposed as a chaperone of retinoid metabolism [34]. The cytoplasmic concentration of CRBP-1 may determine the ability of the cell to accumulate retinol, and thus, serve as a cell regulator for its incorporation [35] [36]. CRBP-1 would be involved in the intermembrane movement of retinol, as well as in the metabolism of both retinyl esters (for storage) and retinal for subsequent activation or catabolism [37].

In vitro experiments show that retinoic acid can up-regulate CRBP-1 expression in adipocytes [38]. Hussmann *et al.* [39] observed that the expression of rat CRBP-1 can be upregulated by retinoic acid. In spleen, lung and testis of rats with 70 days of retinol deficiency, CRBP-1 mRNA decreased. In our results it was observed that the expression of CRBP-1 decreased in the VAD group, whereas in the REF group, the expression of CRBP-1 did not reach the level of the CO group. Our findings demonstrate the connection between CRBP-1 expression and retinoic acid levels. The 5 region flanking CRBP-1 gene is conserved between rat and mouse, and includes a RARE (retinoic acid response element) at 1 kb upstream of the start site of transcription. RARE is activated by RAR (α and β), but not by RARγ1. On the other hand, RARE of CRBP-1 is most effectively activated when RAR and RXR are present. Induction of CRBP-1 transcription by retinoic acid is mediated by the binding of a RAR/RXR heterodimer with a RARE located in a specific promoter region [39].

The accumulation of CRBP-1 promotes the conversion of retinol to retinoic acid [40]. Ghyselinck *et al.*, in a study of CRBP-1 null mice, demonstrated that CRBP-1 is essential for the efficient storage of retinol, but is not essential for retinoic acid synthesis [41]. Moreover, the downregulation of CRBP-1 has been associated with the malignant phenotype, especially in breast and ovarian cancer [29] [42] [43]. In 24% of human breast carcinomas, CRBP-1 expression is decreased; which implies a relationship between cellular homeostasis of vitamin A and breast cancer. The loss of CRBP-1 restricts the effects of endogenous vitamin A on neoplastic mammary gland cells. [29]. Esteller *et al.*, in 2002, suggested that aberrant methylation of the promoter region may be one of the mechanisms underlying the silencing of CRBP-1 in tumor cell lines and in primary tumors [43]. Alterations in CRBP-1 expression and hypermethylation occur frequently in prostate carcinoma, although CRBP-1 hypermethylation is not an early event in this type of cancer [45].

The upregulation of homologous CRBP proteins (CRBP-2 and/or CRBP-3) contribute to the maintenance of the retinoid acid level in the absence of CRBP-1 [46] [47]. However, since CRBP-1 and its homologues normally have different functions, CRBP-2 and CRBP-3 don't restore the total functionality of CRBP-1 [47] [48]. In addition, loss of CRBP-1 is an early event in the progression of breast cancer and is associated with poor prognosis [29]. Loss of CRBP-1 disrupts retinoic acid homeostasis, resulting in breast defects similar to those observed in the early stages of tumorigenesis [49]. These data highlight the role of CRBP-1 as a regulator and also emphasize the essential role of retinoic acid in maintaining adequate breast morphology and the need for strict regulation of the active metabolite of vitamin A. The characterization of CRBP-1 phenotype will help with the development of novel therapeutic strategies for CRBP-1 deficient breast cancer.

A loss of CRBP-1 expression is also associated with the development of less differentiated endometrial carcinomas [50]. CRBP-1 hypermethylation is re-

sponsible for the loss of transcription of CRBP-1 mRNA that takes place in pre-malignant lesions and frequently accompanied by RARβ2 hypermethylation in the same tumors. Moreover, it was observed that a higher dietary intake of retinol was associated with decreased methylation of both genes [43].

In recent years, the role of retinoid signaling along with CRBP-1 has become the subject of several studies in cancer progression. CRBP-1 suppression is associated with a more aggressive phenotype in breast, ovary, and nasopharyngeal cancer. Overexpression of CRBP-1 increases the sensitivity to retinol and reduces the viability of ovarian cancer cells in vitro [51].

Epidemiological studies have suggested an inverse correlation between cancer development and vitamin A consumption [52]. Natural and synthetic retinoids have been shown to inhibit the growth and development of different types of tumors; such as: skin, breast, oral cavity, lung, hepatic, gastrointestinal, prostate, and bladder cancers [53] [54].

8. Conclusions

For this reason, this animal model of virgin rats with 6 months of deficiency in vitamin A can be a study model to observe the different changes that this generates in a virgin mammary gland that despite being in the basal state already presents a deficiency in vitamin A. This gives an excellent field study to observe the metabolic processes such as tumor predisposition, inflammation, among others, where this vitamin is involved.

Acknowledgements

Conflict of Interest

No potential conflict of interest was disclosed.

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