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Assessment of the Relative Dose-Response Test as Indicators of Hepatic Vitamin A Stores in Various Stages of Chronic Liver Disease

Wilza Arantes Ferreira Peres, MD[1]; Gabriela Villaça Chaves, MD[1]; José Carlos Saraiva Gonçalves, MD[2]; Andrea Ramalho, MD[3]; and Henrique Sergio Moraes Coelho, MD[4]

Abstract

Hepatic vitamin A stores should be the best early indicator of vitamin A status because more than 90% of total body vitamin A is stored in the liver. The objective of the present study was to evaluate the hepatic vitamin A stores in all stages of chronic liver disease (CLD), including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). One hundred forty-four patients (age 55.34 ± 9.38 years) were evaluated in a cross-sectional study. Vitamin A nutrition status was analyzed by serum retinol levels and relative dose-response (RDR) method. Patients with cholestasis were excluded from the sample group. Biochemical, clinical, and anthropometric evaluations were performed. Vitamin A deficiency (VAD) was detected in 51.4% of all patients. Patients with adequate levels of serum retinol presented adequate liver vitamin A reserves; in contrast, nearly half the patients with low serum retinol levels presented adequate levels of retinol in the liver, although none of the patients with hepatitis had this condition. Therefore, the effectiveness of the RDR method for evaluating vitamin A nutrition status was limited in patients with cirrhosis and HCC, perhaps due to the advanced age of these patients, since those in the chronic hepatitis group, who were younger, responded adequately to the test. Thus, the RDR method should be modified when applied to later stages of CLD, considering the time and dose of retinyl palmitate supplementation, as VAD may be a risk factor for the progression of the disease. (*Nutr Clin Pract.* 2013;28:95-100)

Keywords

retinol; vitamin A deficiency; hepatitis C; end stage liver disease

The hepatitis C virus (HCV) infection represents the leading cause of liver transplant in Europe and the United States.[1] The pathogenesis of chronic inflammation and fibrogenesis in HCV infection is characterized by an inflammatory and fibrotic process that leads to a progressive evolution from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC).[2] The involvement of oxidative stress in HCV-induced liver damage has been studied. It was reported that reactive oxygen species (ROS) activate hepatic stellate cells, which then lead to hepatic fibrosis and liver disease progression.[3]

In a normal liver, hepatic stellate cells are major storage sites for vitamin A, and following the activation of hepatic stellate cells, a loss of the characteristic stored intracellular vitamin A occurs. Findings from others studies have previously demonstrated that there is a progressive reduction in serum retinol levels from controls to patients with cirrhosis and HCC, so low serum retinol levels may be a risk factor for the development of HCC.[4-6]

Vitamin A deficiency (VAD) is one of the world's greatest malnutrition problems. According to the World Health Organization, Brazil is classified as having a high prevalence of subclinical VAD.[7] Vitamin A participates in several functions that are essential to human health and has gained prominence for its role against ROS, protecting the organism against oxidative stress.[4,8]

Adequate evaluation of vitamin A nutrition status in HCV chronic liver disease is very important since these patients are at risk for oxidative stress and chronic liver disease progression. The hepatic vitamin A stores should be the best early indicator of vitamin A status because more than 90% of total body vitamin A is stored in the liver.[9,10] The widely used serum retinol levels may be a poor reflection of vitamin A nutrition state since previous studies have demonstrated normal levels of serum retinol despite low concentrations of retinol in liver biopsies, indicating VAD.[11] Consequently, other indices may be needed to assess vitamin A nutrition status in patients with HCV chronic liver disease.

From [1]Department of Nutrition and Dietetics, Institute of Nutrition, [2]Department of Medicine, Faculty of Pharmacy, [3]Department of Social and Applied Nutrition, Institute of Nutrition, and [4]Section of Hepatology, University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

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Corresponding Author:
Wilza Arantes Ferreira Peres, Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde–Instituto de Nutrição Josué de Castro, Avenida Brigadeiro Trompovisky, s/nº, Bloco J, 2º andar, sala 07–CEP: 21949-900, Brazil.
Email: wilza@nutricao.ufrj.br.

The relative dose-response (RDR) test is a noninvasive test that allows indirect estimation of the total hepatic stores of vitamin A.[12] It is based on the principle that when liver reserves are depleted, release of stored vitamin A decreases, but synthesis of the retinol-binding protein (RBP), the main transporter of vitamin A, continues. Therefore, when hepatic vitamin A reserves are low, plasma retinol concentration increases markedly 5 hours after an oral loading dose of vitamin A.[13] Consequently, the RDR test may be considered a good functional reference method.

Considering the role of ROS in HCV pathogenesis and the potent action of vitamin A in the fight against ROS, the aim of this study was to evaluate hepatic vitamin A stores in all stages of chronic liver disease (CLD), including chronic hepatitis, liver cirrhosis, and HCC, superimposed on liver cirrhosis.

Materials and Methods

Population

In this cross-sectional study, data were collected prospectively from September 2006 to December 2009 at the University Hospital of the Federal University of Rio de Janeiro, Brazil. The study was approved by the local institutional research ethics committee of the University Hospital and was conducted according to the guidelines in the Declaration of Helsinki. All study participants signed an informed consent agreement.

Patients aged ≥18 years with HCV-related chronic disease (serum HCV-RNA) were eligible for enrollment. Liver cirrhosis diagnosis was based on clinical manifestations and laboratorial testing, as well as ultrasonographic imaging and histological evaluation whenever necessary. Diagnosis of HCC was based on computerized tomography and or magnetic resonance findings and serum α-fetoprotein. All patients in the cross-sectional sample were followed up for at least 17 months. The criteria for exclusion were disabsorptive syndromes, type 1 diabetes mellitus, respiratory or cardiovascular disease, cancer except HCC, cholestasis, chronic renal failure, amyloidosis, pregnancy, chronic and acute alcoholism, use of supplements or drugs containing vitamin A during the past 6 months, and any other etiologic factor besides HCV infection.

Evaluation of Vitamin A Nutrition Status

Assessment of serum retinol. In determining serum retinol concentration, a fasting 5-mL blood sample was taken intravenously from patients on a 12-hour fast. Separation and quantification of the serum retinol were carried out by a validated method for online solid-phase extraction in a Prospekt 2 system (Spark Holland, Emmen, the Netherlands) coupled with high-performance liquid chromatography/mass spectrometry (triple-stage quadrupole Quattro LC system with APCI interface; Micromass-Waters, Wythenshawe, UK). All-trans-retinol (Sigma, St Louis, MO) was used as the reference standard for analysis, as described previously.[6]

In this study, a value of ≥1.05 μmol/L or 30 mcg/dL of serum retinol was considered adequate, with a cutoff point of <1.05 μmol/L or <30 mcg/dL indicating VAD.[14]

RDR method. After an overnight fasting, an initial sample of 5 mL venous blood was taken from a forearm vein. Immediately after, an oral dose of 1500 UI retinyl palmitate (UNICEF, Batch, 948 R.P. Schem Pty Co, Melbourne, Australia) dispersed in 1 mL oil solution was administered to each patient. A few minutes later, a standard breakfast consisting of a cheese sandwich and chocolate milk was given to warrant retinyl palmitate absorption. This meal contains an estimated mean 9.7 g of fat and 378 μg retinol activity equivalents (RAE) of vitamin A. Patients were only allowed to drink water during the 5 hours after the administration of the retinyl palmitate. After 5 hours, a second sample of blood was taken. The samples were processed at the laboratory under minimal or red light to protect light-sensitive compounds from degradation, centrifuged, and frozen at –80°C until analyzed. The results of the RDR test were obtained by using the following formula, as originally described[13]:

$$RDR = A_5 - A_0/A_5 \times 100,$$

where A_0 = basal concentration of retinol and A_5 = retinol concentration in the sample obtained 5 hours after the dose. A positive RDR test ≥20% indicates inadequate vitamin A hepatic stores.[15]

Assessment of Liver Complications and Biochemical Parameters of Liver Function and Liver Damage

The laboratory data collected included serum albumin, total bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), and prothrombin time (PTT), which were measured by previously established standard laboratory methods. The AST:ALT ratio was then calculated for each patient.

Clinical variables recorded were degree of ascites and hepatic encephalopathy based on ultrasound data and clinical evaluation, respectively. Laboratory data and degree of ascites and hepatic encephalopathy were used to calculate the Child-Pugh score. The patients were then stratified as A, B, or C in accordance with the Child-Pugh classification criteria.[16]

Anthropometric Evaluation

Nutrition status was evaluated based on mid-arm muscle circumference (MAMC) and triceps skinfold thickness (TST) to assess muscle protein and adipose tissue, respectively. Mid-arm circumference (MAC) and TST were measured with a measuring tape and a Lange skinfold caliper, respectively. To

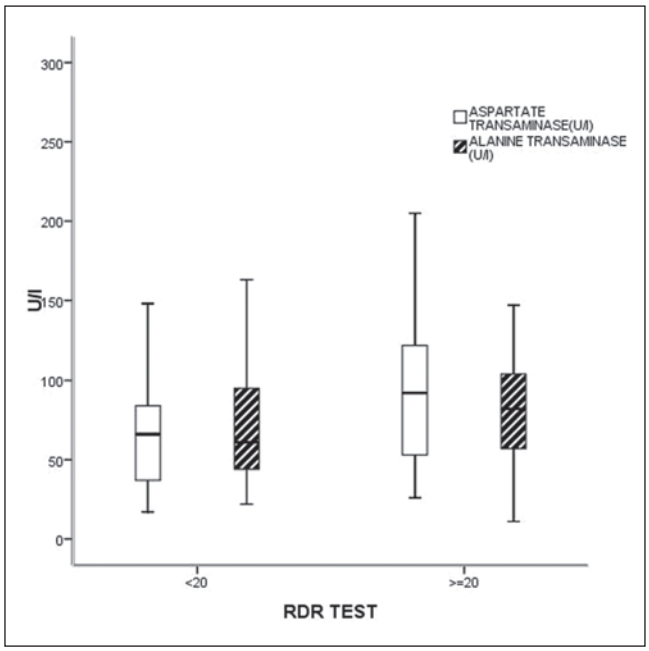

Figure 1. Biochemical parameters of liver damage between the groups with adequate and inadequate liver vitamin A reserves. There are not significant differences between these groups. RDR, relative dose-response.

minimize practical variability, we recorded the average of 3 consecutive measurements for TST. MAMC was calculated according to formulas described by Frisancho[17] based on MAC and TST measurements. Loss of fat and muscle tissue was defined as MAMC and/or TST below the 10th percentile.[18]

Statistical Analysis

The distribution of the referred values was identified as not normal by the adhesion test of the Kolmogorov-Smirnov test.

The Mann-Whitney test was used to compare the numeric variables between the 2 groups. Associations between the categorical variables were performed by Pearson χ^2 test.

Analyses were performed with Statistical Package for Social Sciences software version 16.0 (SPSS, Inc, an IBM Company, Chicago, IL). Significance level was set at $P < .05$.

Results

The sample group was composed of 144 patients, of whom 74 (51.4%) were men. The patients were on average 55.34 ± 9.38 years of age, varying from 33 to 76 years, with 65.3% of the sample group in the 51- to 70-year-old range. Average age of the group with chronic hepatitis (48.7 ± 10.0 years) was significantly lower than that of the patients with HCC-related or non-HCC-related cirrhosis (58.1 ± 8.7 years) ($P < .001$).

The patients were divided into 5 groups according to what stage of the illness they were in: the group with hepatitis comprised 29 patients, the group with cirrhosis was subdivided using Child-Pugh as the criterion (Child-Pugh A, n = 41; Child-Pugh B, n = 31; Child-Pugh C, n = 26), and the HCC group was composed of 17 patients. The characteristics of the study population are shown in Table 1.

Table 1. Characteristics of the Study Population (n = 144).

	No. (%)
Sex	
Female	70 (48.6)
Male	74 (51.4)
Child-Pugh score	
Chronic hepatitis	29 (20.1)
Cirrhosis, Child-Pugh A	41 (28.5)
Cirrhosis, Child-Pugh B	31 (21.5)
Cirrhosis, Child-Pugh C	26 (18.1)
Hepatocellular carcinoma	17 (11.8)
Anthropometric evaluations	
Loss of muscle tissue	12 (8.4)
Loss of fat	9 (6.4)

Regarding serum vitamin A status in the sample group, the patients studied had average baseline serum retinol levels of 1.17 ± 0.91 μmol/L and 1.30 ± 0.89 μmol/L 5 hours after performing an oral vitamin A test, revealing that 51.4% of the sample group had VAD, with baseline serum retinol levels below the 1.05-μmol/L or 30-mcg/dL cutoff point.

RDR testing showed liver reserves of vitamin A to be inadequate (RDR ≥20) in 49 patients, that is, 34.0% of the sample group studied. A significant association was found between vitamin A reserves in the liver and baseline serum retinol ($P < .0001$). Most patients with adequate levels of serum retinol (87.1%) also had adequate liver vitamin A reserves. In contrast, of the patients with low serum retinol levels, 44.6% (ie, nearly half the patients studied) had adequate levels of retinol in the liver, as shown in Table 2.

Our research turned up conflicting results, where patients with adequate stores of vitamin A in the liver had low levels of serum retinol. Of these, 46.1% had Child-Pugh A cirrhosis, 31.8% had Child-Pugh B, 38.8% had Child-Pugh C, and 41.1% had HCC. None of the patients with hepatitis had this condition. No significant association was found between RDR test adequacy and severity of the illness, as shown in Table 3.

No significant differences were found when comparing the averages of the biochemical variables of liver function and liver damage between the groups with adequate and inadequate liver vitamin A reserves (Figure1, Table 4).

No significant association was found in liver vitamin A reserve adequacy between the groups with or without ascites ($P = .08$) and the groups with or without hepatic encephalopathy ($P = .05$).

The prevalence of loss of muscle tissue and fat was low, with 8.4% having muscle tissue loss and 6.4% having fat loss. Taking into account only participants with normal liver retinol reserves and low serum levels (n = 33), 9.1% had loss of muscle tissue and 3.0% had fat loss.

Table 2. Association Between Baseline Serum Retinol Levels and Liver Vitamin A Reserve.

Liver Vitamin A Reserve, %	Baseline Serum Retinol <1.05 μmol/L, No. (%)	Baseline Serum Retinol ≥1.05 μmol/L, No. (%)	*P* Value
Adequate, <20	33 (44.6)	61 (87.1)	.0001
Inadequate, ≥20	41 (55.4)	9 (12.9)	
Total	74 (100.0)	70 (100.0)	

Significant differences were calculated using the Pearson χ^2 test. A significant difference between the groups was considered at $P < .05$.

Table 3. Association Between Vitamin A Reserves in Liver and Disease Severity.

Severity of the Illness	Inadequate Reserves ≥20%, No. (%)	Adequate Reserves <20%, No. (%)	*P* Value
Chronic hepatitis	10 (20.0)	19 (20.2)	
Cirrhosis, Child-Pugh A	12 (24.0)	29 (30.9)	
Cirrhosis, Child-Pugh B	9 (18.0)	22 (23.4)	.60
Cirrhosis, Child-Pugh C	12 (24.0)	14 (14.9)	
Hepatocellular carcinoma	7 (14.0)	10 (10.6)	

Significant differences were calculated using the Pearson χ^2 test. A significant difference between the groups was considered at $P < .05$.

Table 4. Comparison of Medians of the Biochemical Parameters Between the Groups With Inadequate and Adequate Liver Vitamin A Reserves.

Variables	Inadequate Reserves ≥20%, Median (Min–Max)	Adequate Reserves <20%, Median (Min–Max)	*P* Value
Serum albumin, g/L	3.3 (1.9–4.9)	3.5 (1.9–4.0)	.20
Total bilirubin, mg/L	1.3 (0.3–1.4)	1.0 (0.3–7.7)	.10
PTT (second above control)	2.8 (0.1–7.4)	2.2 (0.1–8.8)	.60
AST, U/L	92.0 (26–279)	76.50 (17–268)	.30
ALT, U/L	82.0 (11–278)	74.0 (14–270)	.40
AST/ALT ratio	1.0 (0.1–2.3)	0.92 (0.1–0.4)	.20
ALP, U/L	108 (45–427)	101.0 (20–290)	.70
GGT, U/L	69.0 (12–239)	0.92 (0.1–1.4)	.20

ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; GGT, γ-glutamyl transpeptidase; PTT, prothrombin time. Significant differences were calculated using the Mann-Whitney *U* test. A significant difference between the groups was considered at $P < .05$.

Discussion

Our findings showed that 34% of the patients who took the RDR test had inadequate liver retinol reserves, corroborating the findings of de Paula et al,[19] who found inadequate liver vitamin A reserves in 35.1% of a sample group comprising liver transplant candidates.

Taking into account patients with adequate levels of serum retinol, 12.7% had inadequate reserves in the liver, suggesting that for these patients, serum retinol levels are maintained by mobilizing liver vitamin A reserves, meaning that as the disease progresses, these patients will go on to develop VAD.

According to the RDR method, nearly half the patients showed normal liver vitamin A reserves accompanied by low serum levels of vitamin A. Such a finding may be interpreted in 2 ways: the first possible explanation is that some factors may hinder the release of retinol into the bloodstream, resulting in a drop in serum levels of retinol while liver reserves are adequate. The second possible explanation is unresponsiveness to the RDR test—that is, serum levels of retinol do not rise after supplementation, even when liver vitamin A reserves are inadequate, especially during the more advanced stages of liver disease. This is likely due to an inadequate duration and/or dose of vitamin A administered in testing.

In the first hypothesis, it is possible that changes stemming from liver disease that the patients underwent, such as alterations in RBP, cellular RBP (CRBP), and transthyretin synthesis, may have had an impact on the results. However, some studies have found no significant reduction in RBP synthesis in the midst of liver disease[12,20]; furthermore, a significant number of patients studied in our research did respond to the test. Severe protein and/or calorie malnutrition (PCM) may

lower RBP synthesis and interfere with the mobilization of endogenous vitamin A. However, even in the case of severe PCM, there is a short serum response time to exogenous sources of dietary vitamin A.[15,21] According to research carried out on animals by Underwood,[22] light to moderate PCM does not invalidate the RDR test for assessing inadequate liver reserves. Either way, the low prevalence of loss of fat and muscle tissue found in our research did not seem to have been a factor influencing the lack of response to the test.

Another possible factor is the occurrence of portosystemic shunts in these patients. Schölmerich et al[23] found that patients with portal hypertension may have intra- and extrahepatic shunts, and hence the capacity of their livers to extract zinc may be diminished, based on how zinc is extracted the first time it passes through the liver and that the mineral possibly circulates enterohepatically. Some authors consider zinc important for synthesizing RBP, and lower levels of retinol may correlate with a zinc deficiency.[24,25] This is controversial, however, as Mobarhan et al[12] found that a zinc deficiency was not a limiting factor in RDR test response, and Weismann et al[26] did not find vitamin A nutrition status to recover when zinc-deficient patients were administered supplementation.

The hypothesis on unresponsiveness to the test during the more advanced stages of the disease can be arrived at based on the malabsorption that accompanies liver disease or on the duration and/or dose of vitamin A administered having been inadequate when implementing the test. It is worth noting that patients at an earlier stage of liver disease (ie, patients in the chronic hepatitis group) had adequate serum retinol levels and normal liver vitamin A reserves, suggesting their vitamin A nutrition status and response to the RDR method were satisfactory.

For our research, we excluded patients with cholestasis from the sample group, reducing the chance of possible fat-soluble vitamin malabsorption contributing to a failed response to the RDR method. As for the dose of vitamin A administered and duration in performing the RDR test, the use of a 1500-UI dose of retinyl palmitate to provoke a response within a 5-hour period proved to be sufficient for the classic risk groups (pregnant women, breastfeeding women, and children), but it was insufficient to overcome the limited absorption and liver changes experienced by some patients with cirrhosis, corroborating the view of Mobarhan et al.[12] Furthermore, Bulux et al[27] noted a drop in postprandial clearance of retinyl esters in the circulation of the elderly, recommending a 7-hour RDR duration for those older than 60 years, which is corroborated by Ribaya-Mercado et al.[28] For our research, 63.5% of the sample group was between 51 and 70 years of age. Moreover, the patients with chronic hepatitis—the only group in which 100% of the participants responded adequately to the test—were significantly younger than the rest of the sample group.

Feranchak et al[10] suggest that the functional test for assaying liver vitamin A reserves—RDR—is the most accurate way of evaluating vitamin A nutrition status in the presence of chronic liver disease, since beside the ethical issues involved in carrying out liver biopsies, research shows that biopsies from different parts of the liver return different concentrations of vitamin A,[29] and the presence of fibrosis may artificially reduce the vitamin A content of the liver when calculated by liver weight.[30] Feranchak et al[10] concluded that serum retinol levels are an excellent initial test for tracking vitamin A deficiency in children with chronic liver disease, with a specificity of 90%, recommending that when serum retinol levels are <0.70 μmol/L, the RDR test may be used to confirm deficiency and the need for supplementation, as supplementation may aggravate liver fibrosis in patients with chronic liver disease but who retain normal levels of vitamin A in the liver.

In our research, we noted no significant difference in average biochemical variables relating to liver damage and function between the groups with either adequate or inadequate liver vitamin A reserves. Nor was there an association between liver reserves and the occurrence of ascites or HE. We found no previous research drawing an association between liver vitamin A reserves and the biochemical variables of liver damage, function, and decompensation that we studied in our research. Previous research has demonstrated a relationship between these variables and serum retinol.[6] It could be that the lack of response to the test made it impossible to determine the relationship between liver retinol reserves and such variables.

RDR is considered the best indirect method for early assessment of vitamin A nutrition status, but it has been shown to have its limitations in those with chronic liver disease, as discussed in this study.

In this study, it is concluded that the standard RDR method did not adequately assess liver vitamin A reserves in patients with cirrhosis and HCC, perhaps due to the advanced age of the patients belonging to this group, whereas the chronic hepatitis group, whose members were younger, responded adequately to the test.

Therefore, it is critical that the RDR method of testing be modified for the later stages of CLD, which is also accompanied by a high risk of VDA. We suggest that the time and dose of retinyl palmitate supplementation be adapted, as a lack of vitamin A may be a factor leading to a worsened prognosis and/or may accelerate the progression of the disease by increasing oxidative stress, as well increase the risk of developing HCC.

Moreover, we suggest that future research assess the RBP, given how inadequate vitamin A transport may trigger liver toxicity when supplementation is administered.

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