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The Relative Dose Response Test Based on Retinol-Binding Protein 4 Is Not Suitable to Assess Vitamin A Status in Very Low Birth Weight Infants

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Key Words
Relative dose response test · Vitamin A · Preterm infant

Abstract
Background: The relative dose response (RDR) test, which quantifies the increase in serum retinol after vitamin A administration, is a qualitative measure of liver vitamin A stores. Particularly in preterm infants, the feasibility of the RDR test involving blood is critically dependent on small sample volumes. ***Objectives:*** This study aimed to assess whether the RDR calculated with retinol-binding protein 4 (RBP4) might be a substitute for the classical retinol-based RDR test for assessing vitamin A status in very preterm infants. ***Methods:*** This study included preterm infants with a birth weight below 1,500 g (n = 63, median birth weight 985 g, median gestational age 27.4 weeks) who were treated with 5,000 IU retinyl palmitate intramuscularly 3 times a week for 4 weeks. On day 3 (first vitamin A injection) and day 28 of life (last vitamin A injection), the RDR was calculated and compared using serum retinol and RBP4 concentrations. ***Results:*** The concentrations of retinol ($p < 0.001$) and RBP4 ($p < 0.01$) increased significantly from day 3 to day 28. On day 3, the median (IQR) retinol-RDR was 27% (8.4–42.5) and the median RBP4-RDR was 8.4% (–3.4 to 27.9), compared to 7.5% (–10.6 to 20.8) and –0.61% (–19.7 to 15.3) on day 28. The results for retinol-RDR and RBP4-RDR revealed no significant correlation. The agreement between retinol-RDR and RBP4-RDR was poor (day 3: Cohen's $\kappa = 0.12$; day 28: Cohen's $\kappa = 0.18$). ***Conclusion:*** The RDR test based on circulating RBP4 is unlikely to reflect the hepatic vitamin A status in preterm infants.

Introduction

Vitamin A is required for embryonic and postnatal development as it represents an essential factor for growth, morphogenesis, and epithelial cell differentiation [1]. Therefore, supplementation of vitamin A has gained importance in very preterm infants, as their premature delivery is associated with reduced hepatic vitamin A stores and an increased risk of developing bronchopulmonary dysplasia [1, 2].

The magnitude of the fetal and neonatal hepatic vitamin A store depends on the transplacental transfer of retinol from mother to fetus [3, 4]. This vitamin A transfer increases during gestation and reaches its highest rates during the last trimester of pregnancy [5, 6]. The fetal hepatic synthesis of retinol-binding protein 4 (RBP4), which is the specific transport protein of retinol in the circulation, also increases throughout pregnancy [7, 8]. Hence, in infants born preterm, vitamin A availability may be insufficient to meet the requirements for extrauterine development.

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The serum retinol concentration measurement has limited value for the evaluation of vitamin A status, as it only changes during stages of vitamin A deficiency, and it poorly mirrors the hepatic vitamin A stores. To evaluate an individual's hepatic vitamin A store, the relative dose response (RDR) test has been advocated [9]. This test measures the relative increase in plasma retinol concentrations after administration of vitamin A [10]. This test has been described as a minor invasive measure, in comparison to a liver biopsy, which is the gold standard to assess the hepatic vitamin A status [9, 10]. The RDR test had been used in preterm infants after intramuscular (i.m.) administration of vitamin A [11–13] but, because of the multiple venipunctures and the total blood volume needed for retinol quantification via high-performance liquid chromatography (HPLC), this test is not commonly performed in very low birth weight (VLBW) infants. As an alternative, assessment of the RDR based on serum RBP4 has been suggested, assuming that the circulating retinol is theoretically transported in complex with RBP4 in a 1:1 ratio [11, 14]. Compared to HPLC-based retinol determination, immunoassay-based quantification of RBP4 requires a much smaller sample volume and less complex technical equipment, but it has a slightly higher coefficient of variability [15, 16].

The purpose of the present study was to compare the retinol-RDR and the RBP4-RDR in preterm infants with a birth weight below 1,500 g (VLBW infants) and to evaluate whether the RBP4-RDR represents a useful alternative assay for predicting the vitamin A status in such infants.

Subjects and Methods

Study Protocol

Infants with a birth weight below 1,500 g (VLBW infants) and a gestational age below 33 weeks (n = 63) who were admitted to the neonatal intensive care unit of Charité University Medical Center, Berlin, Germany, between June 2007 and June 2008 were eligible for participation in this study. Further inclusion criteria were the requirement of supplemental oxygen or mechanical ventilation for more than 48 h of life. Infants with congenital malformations of the liver, kidney, or intestine were excluded. The local institutional review board approved the study protocol and written parental consent was obtained for each infant.

Analysis was restricted to infants who had not received postnatal steroids for bronchopulmonary dysplasia.

Infants were fed with their mother's own breast milk or donor milk, enriched with a breast milk fortifier containing vitamin A (Aptamil FMS; Milupa GmbH, Friedrichsdorf, Germany), and/or with preterm formula (Beba preterm formula; Nestlé Deutschland AG, Frankfurt/ Main, Germany). Infants were also given a complex of fat-soluble vitamins intravenously (250 IU/ml vitamin A as retinyl palmitate; Vitalipid Infant; Baxter Deutschland GmbH, Germany) and received i.m. vitamin A supplementation of 5,000 IU water-soluble retinyl palmitate 3 times a week for 4 weeks (Aquasol A Parenteral; AstraZeneca LP, Westborough, Mass., USA). This supplementation regimen has been described by Tyson et al. [13] and is recommended in the medical guidelines for the prevention and treatment of bronchopulmonary dysplasia in preterm infants [17].

Sample Collection and Analysis

Blood samples were collected on the first day of vitamin A supplementation (day 3 of life) and on the last day of the vitamin A supplementation regimen (day 28 of life) immediately before and 5 h after i.m. vitamin A administration to perform the RDR test. Serum was obtained by centrifuging the blood (3,000 *g;* 10 min), and it was stored at –80°C and analyzed within 3 months. The concentrations of retinol were determined by reversed-phase HPLC (RP-HPLC) as described previously, using a serum volume of 100 µl [18]. RBP4 concentrations were measured via an enzyme-linked immunosorbent assay (ELISA), which requires 10 µl serum for quantification [18].

The RDR values were calculated according to the following equation, using the determined retinol concentration of serum samples taken immediately before and 5 h after i.m. vitamin A administration: $RDR\% = [(retinol_{after} - retinol_{before})/retinol_{after}] \times 100$ [9]. Similarly, the pre- and postinjection RBP4 concentrations were used to calculate the RBP4-RDR. The vitamin A status of the infants was assessed according to the cutoff point of 10% for the retinol-RDR as an indicator of sufficient (<10%) or depleted (≥10%) hepatic vitamin A stores [12, 13]. As retinol and RBP4 are transported in the circulation in a molar ratio of 1:1 in normal healthy individuals, we decided to apply the same cutoff value (10%) to the RBP4-RDR.

Statistical Analysis

Statistical analysis was performed using SPSS Statistics, version 17 (SPSS Inc., Munich, Germany). Data are presented as medians and IQR (25th to 75th percentiles). To compare quantified parameters, the Wilcoxon test was used. Statistical differences between bivariate parameters were calculated using the McNemar test. Spearman's equation was used to evaluate nonparametric correlations. Cross tables were used to calculate the specificity and sensitivity of the retinol-RDR and the RBP4-RDR (<10%; ≥10%). Cohen's κ analysis was conducted to determine the agreement between retinol-RDR and RBP4-RDR. A κ value of 1 indicates perfect agreement, whereas a κ value of 0 indicates no agreement. $p < 0.05$ (two-tailed) was considered statistically significant.

Results

Patients

Sixty-three infants were included in this study. They received intravenous fat-soluble vitamins for 9 days (6–13) and a cumulated dose of 1,840 U (1,380–2,530) of vitamin A. The clinical characteristics of these infants are summarized in table 1.

Table 1. Clinical characteristics of the preterm infants included in this study (n = 63)

Characteristics	Values
Gestational age, weeks	27.4 (25.9–28.9)
Male gender	34 (54)
Birth weight, g	985 (746–1,130)
Head circumference at birth, cm	25.0 (24.0–26.5)
SGA infants	7 (11)
IL-6 >100 ng/l on day 3 of life	16 (25)
IL-6 >100 ng/l on day 28 of life	0 (0)
Nosocomial sepsis	5 (8)
IVH ≥grade 2	3 (5)
BPD	15 (24)
ROP total	15 (25)
ROP ≥stage 2	13 (22)

Data are expressed as medians (IQR; 25th to 75th percentiles) or numbers (%). BPD = Bronchopulmonary dysplasia (assessed at 36 weeks' corrected gestational age or at discharge from the hospital, whichever came first); IL-6 = interleukin-6; IVH = intraventricular hemorrhage; ROP = retinopathia prematurorum; SGA = small for gestational age (birth weight below the 10th percentile).

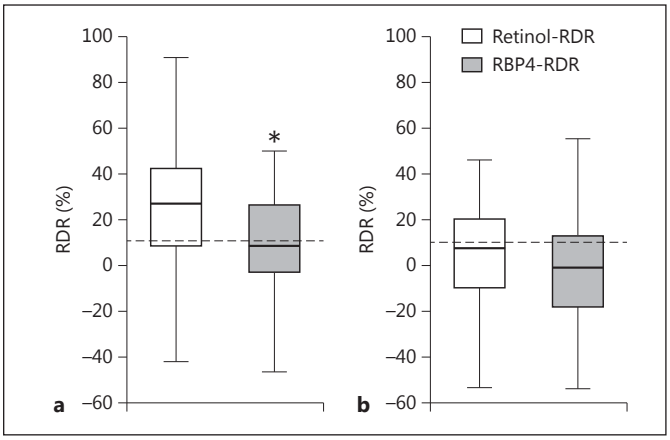

Fig. 1. Box plots representing the RDR after an injection of 5,000 IU retinyl palmitate, calculated with retinol or RBP4 on day 3 of life (**a**) and on day 28 of life (**b**). The dashed line at 10% indicates the RDR threshold that is the cutoff point to categorize hepatic vitamin A stores. Values below 10% indicate replete hepatic vitamin A stores and values equal to/above 10% indicate depleted hepatic vitamin A stores. RDR values were compared using the Wilcoxon test. The asterisk indicates a significant difference between the retinol-RDR and the RBP4-RDR on day 3 of life; $p < 0.001$.

Table 2. Concentrations of retinol and RBP4 in the serum samples of preterm infants before (day 3 of life) and after 4 weeks of vitamin A supplementation with 5,000 IU 3 times a week (day 28 of life)

Parameter	Before the vitamin A injection	5 h after the vitamin A injection	p[a]
Retinol, μmol/l			
Day 3 of life	0.38 (0.29–0.51)	0.52 (0.37–0.68)	<0.001
Day 28 of life	0.60 (0.48–0.78)[b]	0.65 (0.53–0.90)	<0.05
RBP4, μmol/l			
Day 3 of life	0.45 (0.36–0.63)	0.52 (0.38–0.76)	<0.05
Day 28 of life	0.65 (0.43–0.92)[c]	0.67 (0.45–0.97)	0.88

Data are expressed as medians (IQR; 25th to 75th percentiles). [a] Comparing samples taken before and 5 h after i.m. vitamin A supplementation using the Wilcoxon test. [b] Comparing samples taken before the i.m. vitamin A supplementation on days 3 and 28 of life using the Wilcoxon test; $p < 0.001$. [c] Comparing samples taken before the i.m. vitamin A supplementation on days 3 and 28 of life using the Wilcoxon test; $p < 0.01$.

Retinol and RBP4 Concentrations

With regard to changes from the baseline examination (day 3, prior to the first vitamin A injection) to day 28 (prior to the last vitamin A injection), the concentrations of serum retinol ($p < 0.001$) and RBP4 ($p < 0.01$) increased significantly (table 2). The molar ratio of retinol to RBP4 was 0.83 (0.58–1) on day 3 and 0.99 (0.75–1.29) on day 28 ($p < 0.001$). Spearman's rank order correlation coefficient of retinol to RBP4 was 0.38 on day 3 and 0.52 on day 28 ($p < 0.001$).

The i.m. administration of 5,000 IU vitamin A resulted in significantly increased concentrations of retinol on day 3, when the infants received the first i.m. vitamin A injection ($p < 0.001$), and on day 28 (last i.m. vitamin A injection; $p < 0.05$) when comparing samples taken before and 5 h after the vitamin A injection. In contrast, RBP4 concentrations increased on day 3 ($p < 0.05$) but not on day 28 of life, comparing pre- and postinjection concentrations (table 2).

RDR Test: Retinol versus RBP4

The RDR tests, calculated with concentrations of retinol (retinol-RDR) and RBP4 (RBP4-RDR), were performed in 55 VLBW infants on day 3 and in 47 infants on day 28. The reduced number of infants for RDR calculation on day 28 was attributed to insufficient sample volumes.

The median retinol-RDR on day 3 was 27.0% (8.4–42.5%), and on day 28 it was 7.5% (–10.6 to 20.8%) (fig. 1). On day 3, 73.9% of VLBW infants had retinol-RDR values above 10%, indicating vitamin A deficiency. By the 28th

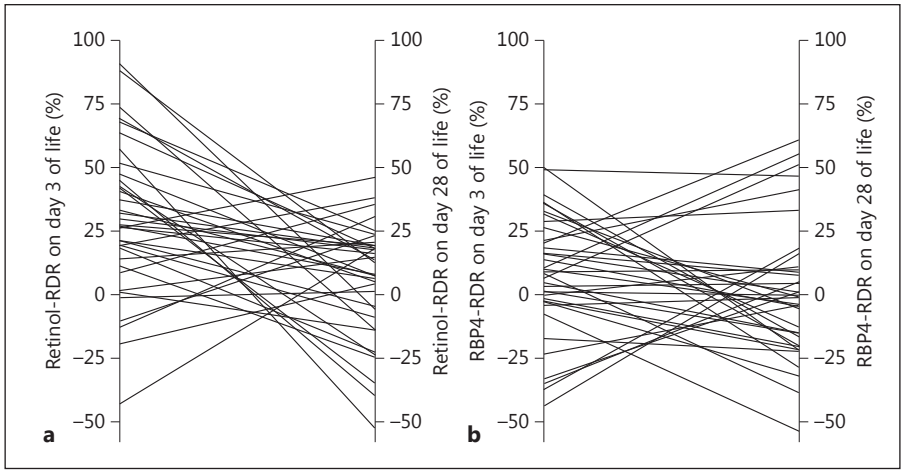

Fig. 2. Individual changes in RDR comparing days 3 and 28 of life in VLBW infants. To determine the RDR, blood samples were taken before and 5 h after i.m. administration of 5,000 IU vitamin A on days 3 and 28. The RDR was calculated with retinol (**a**) and RBP4 (**b**). Between day 3 (first injection) and day 28 (last injection), all infants received i.m. administration of 5,000 IU, 3 times a week.

Table 3. Comparison of the retinol-RDR and the RBP4-RDR to assess vitamin A status in preterm infants on day 3 and day 28 of life

	Retinol-RDR (day 3 of life)			Retinol-RDR (day 28 of life)		
	<10%	≥10%	total	<10%	≥10%	total
RBP4-RDR						
<10%	8 (32)	17 (68)	25 (100)	19 (59.3)	13 (40.6)	32 (100)
≥10%	4 (19)	17 (81)	21 (100)	5 (38.5)	8 (61.5)	13 (100)
Total	12 (100)	34 (100)	46 (100)	24 (100)	21 (100)	45 (100)

Vitamin A deficiency is represented by RDR values above 10%, whereas sufficiency is represented by RDR values below 10%. Values are presented as numbers (%).

day of life, the prevalence of vitamin A deficiency had decreased to 46.7%. The median RBP4-RDR value was 8.4% (–3.4 to 27.9%) on day 3 and –0.61% (–19.7 to 15.3%) on day 28 of life (fig. 1). The prevalence of vitamin A deficiency according to the 10% cutoff of the RBP4-RDR decreased from 45.6% on day 3 to 28.9% on day 28. Figure 2 shows the individual changes in retinol-RDR and RBP4-RDR comparing days 3 and 28 of life.

Spearman's rank order correlation coefficients did not reveal a statistically significant correlation between retinol-RDR and RBP4-RDR (day 3: $r = 0.23$; day 28: $r = 0.10$). Cohen's κ analysis indicated poor agreement between retinol-RDR and RBP4-RDR (day 3 of life: $\kappa = 0.123$; day 28 of life: $\kappa = 0.177$). A limited sensitivity and specificity to classify the vitamin A status in VLBW infants has been calculated for the RBP4-RDR test. In detail, the sensitivity (the probability of the RBP4-RDR to correctly indicate a sufficient vitamin A status in the study population) was 50% on day 3 and 61.9% on day 28 of life, respectively. The specificity (the probability of the RBP4-RDR to identify those infants with a vitamin A deficiency) was 33.3% on day 3 and 20.8% on day 28 (table 3).

Neither retinol-RDR nor RBP4-RDR was associated with clinical outcomes such as bronchopulmonary dysplasia, retinopathy of prematurity, sepsis, or intraventricular hemorrhage.

Discussion

When comparing the retinol-RDR and the RBP4-RDR to assess vitamin A status in VLBW infants, the RBP4-RDR is less sensitive and specific than the retinol-RDR. Regarding the retinol-based RDR and the 10% cutoff, 74% of the VLBW infants were categorized as vitamin A deficient on day 3 of life. Moreover, the study results show that about 50% of the VLBW infants had a low vitamin A status according to the retinol-RDR test on day 28, de-

spite vitamin A supplementation for 4 weeks. Concerning the RBP4-RDR, this test underestimates vitamin A deficiency, compared to the retinol-based RDR test, both on day 3 and on day 28 of life.

For several years, it has been suggested to supplement VLBW infants with vitamin A if they need artificial ventilation, continuous positive airway pressure, and/or additional oxygen for more than 48 h of life to prevent bronchopulmonary dysplasia [8]. To properly supplement VLBW infants with vitamin A, their vitamin A status should be assessed before the intervention and controlled during the supplementation period. However, for VLBW infants, the methods to assess vitamin A status are limited [19]. In the present study, the RDR test, calculated either with retinol or with RBP4 values, has been used to assess vitamin A status. Retinol determination via HPLC requires a relatively large blood sample volume that limits this application in VLBW infants. Alternatively, the RBP4-RDR had been suggested as a surrogate marker to determine vitamin A status because retinol and RBP4 are supposed to be released from the liver in a 1:1 molar ratio [11, 20–22]. The advantages of RBP4 over retinol quantification (RP-HPLC) are a 10× lower sample volume and the need for less complex technical equipment for determination by ELISA.

The retinol-RDR values determined in the present study add to the results described by Ambalavanan et al. [12] and Tyson et al. [13]. In both studies, the vitamin A regimen used in VLBW infants was similar to that of the current study. The authors described proportions of 27 and 34%, respectively, of premature infants who were vitamin A deficient even after recurring vitamin A supplementation. Studies that focused on RBP4 as an indicator of retinol status yielded variable results [20, 21]. Fujita et al. [20] assessed the RBP4-RDR as a potential substitute measure of vitamin A status in lactating women. In contrast, Shenai et al. [14, 22] described RBP4 as a useful parameter for detecting the vitamin A status in VLBW infants. However, the latter study [22] was designed not to compare the retinol-RDR and the RBP4-RDR but to predict bronchopulmonary dysplasia.

The present study also revealed negative retinol-RDR values, indicating a reduction of serum retinol concentrations 5 h after i.m. vitamin A administration. This observation seems unexpected, but it has also been reported by others [13, 19, 23]. Possible causes of negative retinol-RDR values may be a preexisting adequate vitamin A status, the abundant availability of vitamin A after i.m. vitamin A administration, and alterations of the hepatic RBP4 secretion, which affects the values of retinol- and RBP4-RDR. The RDR test presupposes a fasting condition before vitamin A supplementation. Since VLBW infants need frequent feeding [24], a fasting period prior to vitamin A substitution cannot be applied to this population.

Moreover, the weak correlation of serum retinol and RBP4 concentrations indicates an imbalance of serum retinol and RBP4 concentrations. These conditions might originate in the preterm delivery and immaturity of the organs of VLBW infants. In particular the liver, as a central organ of RBP4 synthesis (retinol-free RBP4, apo-RBP4), causes inadequate synthesis and release of this protein bound to retinol (holo-RBP4) [25]. Next to the liver, kidney integrity modulates vitamin A homeostasis. A low glomerular filtration rate, which accounts for an increase in serum RBP4 concentrations, and imperfect reabsorption of RBP4 in the proximal tubule by megalin and cubilin [26], does modulate the RBP4 serum concentration. Reduced reabsorption of RBP4 may account for the weak correlation of retinol and RBP4 concentrations and the lower prevalence of vitamin A deficiency predicted by the RBP4-RDR in comparison to the retinol-RDR in this group of infants.

Taken together, the RBP4-RDR requires a lower blood volume, but it is not applicable to VLBW infants because of its low sensitivity and specificity compared to the retinol-RDR. As i.m. vitamin A substitution is an invasive procedure, a valid test is required to initially assess the circulating retinol concentration, to control the supplementation, and for proper substitution in VLBW infants as opposed to an orally administered dose for the RDR test. As the RBP4-RDR does not seem to be appropriate for VLBW infants, a testing procedure using low sample volumes is of particular interest for neonatologists. Further research in this field is needed focusing on the functional integrity of the liver and the kidneys and their impact on the serum vitamin A status in VLBW infants.

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