

## Association of Vitamin A and Zinc Status with Altered Intestinal Permeability: Analyses of Cohort Data from Northeastern Brazil

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### ABSTRACT

To examine the association of intestinal barrier function with vitamin A deficiency and whether supplementation of micronutrients improves intestinal function and/or linear growth, height-for-age z-score (HAZ), concentrations of serum retinol and zinc, and intestinal permeability were determined in a cross-sectional sample of 75 children in northeastern Brazil. Effects of vitamin A and supplementation of zinc on intestinal permeability and growth were also determined comparing results before and after treatment in 20 children and age-matched controls. Lactulose:mannitol (L/M) permeability ratios inversely correlated with serum retinol concentrations ( $r=-0.55$ ,  $p<0.0005$ ). Increased L/M permeability ratios with reduced concentrations of serum retinol were predominantly attributable to lower absorption of mannitol ( $r=0.28$ ,  $p=0.02$ ). L/M permeability ratios ( $p=0.001$ ) and HAZ scores ( $p=0.007$ ) improved with supplementation. It is concluded that impaired intestinal barrier function and linear growth shortfalls improve following supplementation of vitamin A and zinc in this setting.

**Key words:** Infant nutrition disorders; Child nutrition disorders; Diarrhoea, Persistent; Diarrhoea, Infantile; Vitamin A; Vitamin A deficiency; Zinc; Zinc deficiency; Intestinal permeability; Infant nutrition; Child nutrition; Infant growth; Child growth; Cross-sectional studies; Brazil

### INTRODUCTION

Diarrhoea and malnutrition are major public-health problems in developing countries; the interactions between them are synergistic, bi-directional, and complex

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(1-5), compounding a 'vicious cycle' (6). In this vicious cycle, small intestinal injury, one of the consequences of diarrhoea (7-10), is largely responsible for malnutrition (10,11), which, in turn, both exacerbates and predisposes to diarrhoea (1,3,10,12). Furthermore, vitamin A and/or zinc deficiency is common in malnourished children, which has become a focus of interest. Some studies in animal models have shown the relationships between intestinal mucosal permeability and vitamin A (13,14) and zinc (15). It is, thus, plausible that supplementation of vitamin A and/or zinc in children may help break the chain of this vicious cycle.

In a previous intervention study we conducted in Brazil among children at risk of persistent diarrhoea,

the average duration of diarrhoeal episodes decreased significantly with the oral administration of a single large age-adjusted dose of vitamin A (16). Other studies have demonstrated the effects of vitamin A in reducing childhood mortality from diarrhoea (17-19) and in improving linear growth (20,21). Likewise, it is demonstrated that supplementation of zinc reduces diarrhoeal incidence (22,23) and severity (24,25), and enhances the growth of stunted infants (26,27). However, the mechanisms for these effects remain uncertain.

The dual sugar permeability test is an indicator of small bowel function (28). This test directly measures the ability of two non-metabolized sugar molecules—mannitol and lactulose—to permeate the intestinal mucosa and be excreted in the urine. The larger disaccharide lactulose is poorly absorbed unless the paracellular tight junctions between adjacent enterocytes are disrupted, whereas the smaller monosaccharide mannitol passively diffuses through the enterocyte membrane and is a reflection of total absorptive surface area. Thus, urinary lactulose and mannitol reflect intestinal barrier disruption and absorptive surface area respectively.

The present study was designed to examine the correlation of intestinal permeability with concentrations of serum retinol and zinc and to determine whether or not there are protective effects of supplementation of vitamin A and zinc on intestinal permeability in children with a history of persistent diarrhoea. Using height-for-age (HAZ) score as an indicator of nutritional status and linear growth, we also examined the effects of vitamin A and zinc treatment on childhood growth.

## MATERIALS AND METHODS

### Study area and subjects

Children included in the present study are part of an ongoing cohort study initiated in 1989 in the urban shantytown in Goncalves Dias (population approximately 1,800) in the city of Fortaleza (population approximately 2.2 million) in northeastern Brazil. The study protocol and informed consent were approved by the University of Virginia and the Federal University of Ceará Human Investigation Committees.

In total, 75 children aged 2-97 months (median=33 months) had sera collected for determinations of serum retinol and zinc and had intestinal permeability studies done as part of a study on breast-feeding and intestinal

function. The cross-sectional portion of the present investigation utilized these data to retrospectively assess the relationship between serum zinc and retinol and dual sugar permeability. Results of a preliminary analysis of the initial 30 children in this study have been reported earlier (29).

A subgroup of 20 cases were selected for longitudinal assessment of the effects of supplementation of vitamin A and zinc. These children were defined as those who had a history of at least one episode of persistent diarrhoea or weight-for-age (WAZ) score less than  $-0.5$  in 1997. After obtaining informed parental consent according to the World Health Organization (WHO) guidelines, a single oral dose of vitamin A (100,000 IU for children aged less than one year; 200,000 IU for older children) was administered to this group of children. At the same time, they also received a two-week course of 20 mg of zinc as zinc sulphate per day. These data permitted longitudinal data comparisons of intestinal function in children before and after supplementation of vitamin A and zinc.

During September 1995–April 1998, the 20 children and their age- ( $\pm 6$  months) and HAZ score ( $\pm 0.5$ )-matched neighbourhood controls had height measured at quarterly intervals. The HAZ scores were calculated using anthropometric software of Epi Info 6.04 (CDC, Atlanta and WHO, Geneva, Switzerland).

### Assessments of serum retinol and zinc

Serum zinc concentrations were measured using atomic absorption spectrophotometry at American Medical Laboratories, Inc. (Chantilly, Virginia).

Serum retinol was measured using reverse phase high-performance liquid chromatography (HPLC). This method employs an internal standard consisting of a known mass of retinyl acetate added in 0.5 mL of ethanol to 0.5 mL of serum. The serum samples were denatured in ethanol (1:1 v/v), and retinol was extracted into 2 mL of HPLC grade hexane. Following one backwash of the hexane extract with 0.5 mL of deionized water, the hexane was evaporated to dryness under a gentle stream of N<sub>2</sub>, and the extracted retinol was redissolved in 40  $\mu$ L of benzene and analyzed by reverse phase HPLC. The amount of retinol present in each extracted serum sample was quantitated by comparisons of the integrated areas under the HPLC peaks with a standard curve relating integrated peak area with known masses of retinol and internal standard retinyl acetate prepared

using published extinction coefficients for retinol and retinyl acetate. Retinol was provided by Dr. P. Sorter (Hoffmann-LaRoche, Inc., Nutley, NJ). Retinyl acetate was purchased from Eastman Kodak, Inc. (Rochester, NY). All retinol analyses were performed by the same individual. The within-assay and between-assay coefficients of variations for this assay were respectively 3% and 8%. The lower limit of detection for this assay was 0.02  $\mu\text{mol/L}$ . To assure the accuracy of our serum retinol measures, we evaluated retinol concentrations of a control sera obtained from a volunteer in our laboratory. Aliquots of this control serum were stored at  $-70\text{ }^{\circ}\text{C}$  and measured at regular intervals throughout the study period. At no point did the retinol concentrations determined for this control serum differ by more than 16% (twice the between-assay coefficient of variation for our retinol assays; see above) from the mean. Retinol was separated by reverse phase HPLC on a 250x4.6-mm Beckmann Ultrasphere C18 (5  $\mu\text{m}$ ) column (Beckmann Instruments, Inc., Fullerton, CA) using a mobile phase consisting of acetonitrile:methanol:methylene chloride (70:15:15 vol/vol) at a flow rate of 1.8 mL per minute. The running column was preceded by a C18 guard column. Retinol was detected at 325 nm using a Waters 996 Photodiode Array UV absorbance monitor (Waters Inc., Milford, MA).

#### Urinary lactulose/mannitol

To evaluate small intestinal permeability to lactulose and mannitol, urinary excretions of lactulose and mannitol of participating children were measured by HPLC. For the permeability test, an oral dose of 5 g lactulose and 1 g mannitol in a 20-mL solution was followed by a five-hour urine collection. One mL of 20% (w/v) chlorohexidine was added to each sample as a preservative. Lactulose (4-O-D-galactopyranosyl-D-fructofuranose) was obtained from Duphar B.V. (The Netherlands), and mannitol (6-O-D-galactopyranosyl-D-glucopyranose) was obtained from Quimiobras, Ind. (Quimicase, RJ, Brazil). The total urine volume was recorded upon completion of the test, and a 20-mL aliquot was stored at  $-20\text{ }^{\circ}\text{C}$  until analysis. Concentrations of urinary lactulose and mannitol were measured by HPLC as described by Barboza *et al.* (7).

#### Statistical methods

Urine lactulose and mannitol data were converted using a square root transformation to normalize the distribution. Pearson's and partial correlation analyses were done to determine the correlations between serum vitamin A or zinc and urine lactulose/mannitol (L/M) ratio. Paired

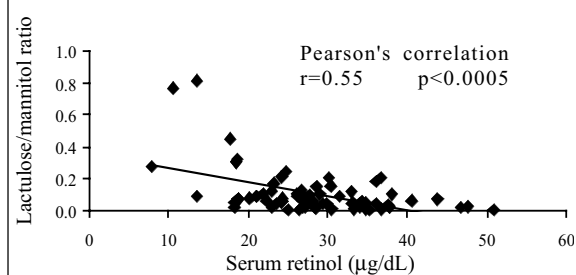
*t*-tests were used for comparing the levels of urine lactulose and mannitol before and after the treatment. The above analyses were performed with SPSS 10.0 (SPSS, Inc., Chicago, USA).

A repeat measures model was used for examining the effects on linear growth of children approximately 12 months after supplementation of vitamin A and zinc for cases and for the two sets of controls. In fitting this first-order autoregressive model AR(1), it was assumed that the correlations decrease exponentially with the distance between measurements. All estimates in this analysis were obtained using restricted maximum likelihood methods (REML). The AR(1) model was fitted in SAS 6.21 (SAS Institute, Cary, NC, USA). All tests were 2-tailed, and the significance level was 0.05.

### RESULTS

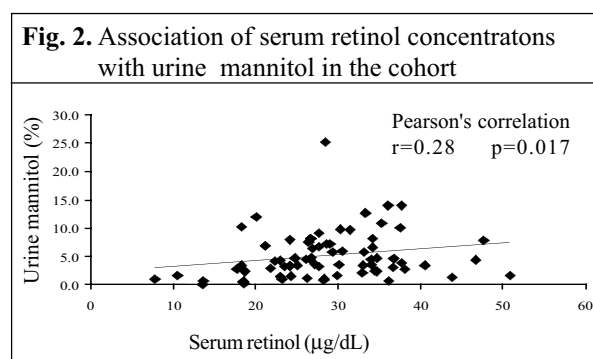
L/M ratios correlated inversely with serum retinol concentrations ( $r=-0.55$ ,  $p<0.0005$ , by Pearson's correlation) (Fig. 1).

**Fig. 1.** Association of serum retinol concentrations with urine lactulose/mannitol ratios in the cohort



Mannitol absorption correlated significantly with serum retinol concentrations ( $r=0.28$ ,  $p=0.017$ ) (Fig. 2), and to a lesser extent, lactulose absorption tended to correlate inversely with serum retinol levels ( $r=-0.22$ ,  $p=0.063$ ). These effects held among children with low retinol levels ( $<0.7\text{ }\mu\text{M/L}$ ) ( $n=46$ ) after stratifying by the retinol levels, i.e. the correlation of reduced mannitol ( $r=0.44$ ,  $p=0.002$ ) and, thus, higher L/M ratios ( $r=-0.62$ ,  $p<0.001$ ) with lower retinol levels held within the subset with subnormal serum retinol concentrations. In the subset of children with serum retinol concentrations within the normal range ( $n=29$ ), however, lactulose was significantly correlated inversely with serum retinol status ( $r=-0.42$ ,  $p=0.024$ ), whereas mannitol was not ( $r=-0.23$ ,  $p=0.225$ ). No statistically significant correlations were found between age and vitamin A status, zinc status, or urinary

L/M measurements (n=75, 53% females; age 2-97 months, median=33 months). The means and standard deviations of the serum retinol, urine lactulose, mannitol, and L/M ratios in the 75 children were  $28.3\pm 8.3$ ,  $0.5403\pm 0.2863$ ,  $2.069\pm 0.8800$ , and  $0.2883\pm 0.1623$  respectively.



Serum zinc levels of 51 children were assayed. In contrast to serum retinol, using bivariate analyses, serum zinc levels did not correlate with lactulose, mannitol, or L/M ratios (partial  $r=0.02$ ,  $0.05$ ,  $-0.10$ ;  $p=0.895$ ,  $0.751$ ,  $0.495$ ), nor did zinc correlate with lactulose, mannitol, or L/M ratios using partial correlation analysis ( $r=0.04$ ,  $0.03$ ,  $-0.06$ ;  $p=0.779$ ,  $0.858$ ,  $0.689$ ). When we examined partial correlations between serum retinol and urinary L/M ratios, there was a trend for retinol to correlate with L/M ratios even when controlling for serum zinc levels (partial  $r=-0.14$ ,  $p=0.071$ ).

When correlation analysis was done on 20 children receiving vitamin A and zinc supplements, we found the same relationships that we have determined in the 75 children, a negative correlation between serum retinol concentrations and urinary L/M ratios ( $r=-0.498$ ,  $p=0.025$ ), and a positive correlation between serum retinol concentrations and urinary mannitol levels ( $r=0.508$ ,  $p=0.022$ ).

However, such effects were not found between serum zinc concentrations and mannitol, lactulose, or L/M ratios ( $r=-0.025$ ,  $-0.391$ ,  $-0.335$ ;  $p=0.916$ ,  $0.088$ ,  $0.148$ ), nor were effects of zinc seen by partial correlation analysis when controlling for serum retinol levels (partial  $r=-0.361$ ,  $-0.445$ ,  $-0.118$ ;  $p=0.129$ ,  $0.056$ ,  $0.629$ ). After controlling for serum zinc levels, however, there was still a positive correlation between serum retinol concentrations and mannitol absorption (partial  $r=0.596$ ,  $p=0.007$ ), but not between serum retinol concentrations and urinary lactulose levels or L/M ratios (partial  $r=0.230$ ,  $-0.419$ ;  $p=0.344$ ,  $0.074$ ).

Paired *t*-test showed that statistical improvements occurred in L/M ratios ( $0.2\pm 0.12$  vs  $0.28\pm 0.12$ , for pre- vs post-supplementation,  $t=3.738$ ,  $p=0.001$ ), but not mannitol and lactulose intestinal permeability indicators (Table 1).

**Table 1.** Demographic characteristics and serum retinol, zinc and urine mannitol and lactulose measurements in the study cohort

Characteristics (n=75)	Baseline level	
Median age (months)	33 (range: 2-97)	
Sex		
Male	35 (47%)	
Female	40 (53%)	
Serum retinol	$28.34\pm 8.31$	
Urine mannitol	$2.07\pm 0.88$	
Urine lactulose	$0.54\pm 0.29$	
Lactulose/mannitol ratio	$0.29\pm 0.16$	
	n=20	
	Pre-treatment	Post-treatment
Age (months)	$62.5\pm 20.5$	
Sex		
Male	7 (35%)	
Female	13 (65%)	
Serum retinol	$28.55\pm 7.60$	
Serum zinc	$692.85\pm 88.52$	
Urine mannitol	$2.23\pm 0.84$	$2.28\pm 0.69$
Urine lactulose	$0.56\pm 0.22$	$0.46\pm 0.22$
Lactulose/mannitol ratio	$0.28\pm 0.12$	$0.19\pm 0.07^*$
	* $t=3.738$ , $p=0.001$	

To compare HAZ scores 3 months before treatment with scores 3, 6, and 9 months after treatment, analyses using the AR(1) model were performed for the 20 matched case-controls, using time and group assignment as two factors. The effect of group had no statistical significance ( $F=0.02$ ,  $p=0.8841$ ), showing the comparability between the group of cases and controls. Time and time by group analyses reached statistical significance ( $F=3.56$ ,  $4.29$ ;  $p=0.0168$ ,  $0.0067$ ), indicating that HAZ scores improved following treatment (Table 2).

## DISCUSSION

An increased L/M ratio has been observed in our cohort study conducted in northeastern Brazilian children with diarrhoea compared to those without diarrhoea (7). The present study was conducted to examine the potential role of supplementation of vitamin A and zinc in promoting or maintaining intestinal epithelial integrity. Analyses of serum retinol and zinc levels showed a significant inverse correlation between serum retinol concentrations and urinary L/M ratios, but not between zinc and urinary indicators. This effect was accounted for predominantly by higher mannitol absorption with higher serum retinol levels, which hold even when the

effects of zinc were controlled. The potential interaction between vitamin A and zinc was another focus of our

**Table 2.** Demographic characteristics and HAZ scores of 20 case-controls at quarterly intervals

Characteristics	Case	Control
No. of children	20 (7M, 13F)	20 (5M, 15F)
Age (months)	62.5±20.5	61.0±20.9
HAZ0	-1.01±1.26	-0.78±1.05
HAZ1	-0.75±1.02	-0.86±1.02
HAZ2	-0.71±0.95	-0.78±1.04
HAZ3	-0.62±1.03	-0.73±0.98

Tests of fixed effects [(AR(1) model)]: group (case and control):  $F=0.02$ ,  $p=0.8841$ ; time:  $F=3.56$ ,  $p=0.0168$ ; time by group:  $F=4.29$ ,  $p=0.0067$   
 F=Female; M=Male  
 HAZ: Height-for-age z scores  
 HAZ 0, 1, 2, and 3: HAZ measured at different times, 3 months before and 3, 6, and 9 months after the supplementation

study. However, zinc status did not correlate with urine L/M irrespective of whether or not we controlled for the effects of vitamin A. However, in the group of children with serum retinol levels within the normal range, such a relationship was not found as it was lactulose instead of mannitol that improved in this group. Since mannitol uptake/excretion is an indicator of overall villous surface area, this suggests that either villous surface area is compromised as a result of vitamin A deficiency and/or vitamin A status is compromised in children who have decreased villous surface area. Either possibility, and quite likely both, could contribute to the synergy observed between malnutrition and diarrhoeal disease. Although decreased intestinal villous height/crypt depth ratio and increased L/M ratio have been documented in children with diarrhoea and malnutrition (7,9), we cannot conclude on the involvement of specific micronutrients in these abnormalities. After supplementation of vitamin A and zinc, statistically significant reduction in the L/M ratio suggests that the supplementation could result in an improvement in these indicators of intestinal integrity.

Because this analysis cannot determine the direction of causality, we next tested whether supplementation of vitamin A and zinc would result in an improvement in these indicators of intestinal integrity. We found that supplementation of vitamin A and zinc resulted in significant reductions (improvements) in the L/M ratio. The changes in the L/M ratio were largely due to a decrease in lactulose absorption, which may be predominantly an effect of zinc, based on the evidence that the reductions of lactulose excretion, but not

increased mannitol absorption, were significantly influenced by zinc supplementation in Bangladeshi children with acute and persistent diarrhoea (30). A trend towards improved mannitol absorption was also seen in four children who only received vitamin A (data not shown). Thus, our findings, taken with previous reports, suggest that zinc supplementation may decrease (improve) lactulose excretion (i.e. reduce barrier disruption), whereas vitamin A treatment may improve mannitol excretion (i.e. enhance absorptive surface area). In addition, the vitamin A status in our children also correlated best with mannitol absorption. Thus, vitamin A and zinc may have different predominant effects on intestinal permeability that are complementary and may be synergistic.

The effect of vitamin A and zinc treatment on linear growth in this study is consistent with the results from trials on vitamin A supplementation conducted in Indonesia and Tanzania (20,21), and zinc supplementation in Bangladesh (30) and Ethiopia (27). Supplementation of vitamin A modestly improved overall growth in vitamin A-deficient children. In addition, the sustained effect indicated that the duration of the effect on growth following a single dose of vitamin A can last for at least nine months. Indeed, the dosing interval of 4-6 months in recent supplementation studies (17,18,20) appears to be appropriate. Although we also stratified by zinc in fitting the autoregressive model, no interaction of group by time was found. HAZ improved at the same rate regardless of whether zinc was supplemented, but this does not necessarily rule out an impact of zinc on linear growth due to the small sample size.

Overall, our results show the correlation of vitamin A and zinc with intestinal absorptive function and suggest an impact on children's growth. However, a small number of cases who received treatment limits the opportunity for subgroup analysis. Additionally, several other questions remain unanswered. First, what would be the effects of vitamin A or zinc administration alone on intestinal permeability and linear growth? Second, are there any interactions between the effects of vitamin A and zinc supplementation on intestinal permeability and linear growth? Answers to these key questions require further studies on vitamin A and zinc and perhaps other nutrient interactions. We conclude that reduced levels of serum vitamin A correlate with impaired intestinal function and that vitamin A with zinc may help improve intestinal function and growth, and thus help break the vicious cycle of diarrhoea and enteric infections with malnutrition and impaired development.

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