

Full Length Research Paper

Malaria as an underlying cause of *Hypovitaminosis A*: results of HPLC retinol assessment among preschool children in Senegal rural areas

Diatla A¹, Cissé F¹, Traoré S², Ndiaye B³, Kaïmba LC⁴, Diallo F¹, Sarr GN¹, Sall ND¹, Touré M¹

¹Laboratory of Biochemistry and Molecular Biology, Faculty of Medicine, Pharmacy and Odontology, Cheikh Anta Diop University of Dakar, Senegal

²Laboratory of Chemistry, Faculty of Sciences and Technology, Cheikh Anta Diop University of Dakar, Senegal

³The Non-Governmental Organization World Vision, Dakar, Senegal

⁴Laboratory of Medical Analysis, Regional Hospital of Ziguinchor, Senegal

Accepted March 18, 2013

Vitamin A deficiency and malaria are a serious public health problem in Africa. Both conditions form a vicious circle in which vitamin A deficiency is frequently found in children suffering from malaria. However, the anterior or secondary character of vitamin deficiency in relation to malaria infection is still unknown. To unmask the isolated negative effect of malaria on retinol plasma level, in this study we have correlated vitamin depletion and malaria infection in children who previously received effective vitamin A supplementation. The results of the HPLC analysis of retinol serum levels indicate vitamin A significantly lower in malaria patients compared to those with negative thick blood smear ($p = 0.004$). In addition, the rates appeared relatively lower in the age group between 12 and 35 months ($p < 10^{-5}$), more in female subjects compared to those of males ($p = 0.04$). Together, these results indicate the interest in combining malaria control and protection against vitamin A deficiency in developing countries. Vitamin supplementation could then target vulnerable populations and more during periods of high malaria endemicity.

Keywords: HPLC, Children, Vitamin A deficiency, Malaria.

INTRODUCTION

Avitaminosis is highly present in areas where malaria prevalence is unacceptable high (Shankar, 1995; Marsh, 1998). The both affections form a vicious circle (Sanjoaquin et al., 2009). A low serum retinol concentration is commonly found in children suffering from malaria (Das et al., 1996; Hautvast et al., Sauerwein et al., 1998; Raza et al., 2009) but it is not certain whether this represents pre-existing vitamin A deficiency. So, this present study is undertaken to test whether malaria has a negative effect on vitamin A homeostasis among preschool children living in Senegal rural areas.

MATERIALS AND METHODS

Subject enrolment

The sampling has involved 312 under 5 year-old children.

These children received vitamin supplementation carried out by the Senegalese State and World Vision (NGO) in the health districts of Mekhé (Thies Region) of Kebemer (Louga Region) Bambey (Diourbel Region) and Kounghoul (Kaolack Region) in Senegal. Short stays in the study areas and the lack of consent of the people targeted were the main criteria for exclusion. Informed consent from parents was obtained for participation in the study. They were paired one another according to age, sex, and according to nutritional status.

*Corresponding Author Email: adiatta@refer.sn

Exploration of the plasma retinol levels

We collected in dry tubes 5ml-blood, by venipuncture in the antecubital fossa. Then the blood was separated into sera by centrifugation at 3000 rev / min for 10 minutes. All the sera were protected from photo-degradation and the deleterious effects of oxygen by adding two drops of anti-oxidant (Butylated hydroxytoluen 2.6-ditert-butyl – 4-methylphenol). The tubes containing sera were also wrapped in aluminum foil. The samples thus treated were frozen at -20°C until extraction and determination of vitamin A levels.

The vitamin A has been extracted relying on the difference of solubility in the polar (ethanol) and organic (hexane) phases. Serum (0.5 ml) was added in 1.5 ml of 95% ethanol / hexane in a ratio of 1:2 (v / v). After vortexing for 2 minutes, the mixture was centrifuged at 2500 rev / min for 10 minutes. The supernatant (hexane extract) was decanted and evaporated to dryness. The resulting pellet was resuspended in 300 μl of acetonitrile and pooled with internal standard (retinyl-acetate). Then the retinol was assessed by RP-HPLC according to the analytical process advocated by Underwood BA (Underwood, 1990)

The Chromatography system used was the Jasco 880-PU[®], fitted with a 50 μl injection sample loop, a UV-880 / UV-875[®] detector, a Spectra-Physics integrator, 4270[®] model and a Spherisorb 5-phenyl column[®], 150 cm x 0.46 cm (Prolab[®], France). Briefly, the biological samples (standard vitamin at different concentrations and extracts) were analyzed by isocratic reverse-phase HPLC. The mobile phase was composed of a mixture in the proportions of 90/10 (v / v) acetonitrile / water HPLC grade with a flow rate of 1ml/minute. The elution peaks were detected at the wavelength of 325 nm.

For each standard solution, the concentration was correlated to the elution peak area in the RP-HPLC chromatogram. This process led to the establishment of a curve calibration from which the vitamin A content of each extract was derived. The tests were repeated in order to assess the reproducibility of the chromatographic method.

Plasmodium detection in blood

Blood sampling was systematically performed among the study population. Blood was collected by pricking with a lancet, at the usual sites, namely the finger, the earlobe, and for newborns at the toe or heel. A large drop of blood was deposited in the center of the glass slide, and was regularly defibrinated turning the tip of a lancet into the drop of blood for two minutes. It was evenly spread over the blade. After drying at room temperature, blood was dehaemoglobinized using distilled water for 3 to 10 minutes, until the mixture was clean. This was followed by freshly prepared Giemsa stain solution (3 drops per 2

ml of buffered distilled water) for 20 minutes. After washing in water, the preparation was dried before light microscopy was performed for plasmodium. Then Giemsa-field stained thick was examined with microscopy to identify presence of plasmodium.

Ethics approval

Approval for the study was obtained from the Ministry of Health and Prevention and ethical committee. Written informed consent for participation was obtained from parents or from other legally responsible person.

Statistical analysis method

The results were expressed as means and standard deviations. For the comparative study of averages, we used the Student t-test. Differences between two means were considered significant when $p \leq 0.05$.

RESULTS

In our study, the retinol assessment by reversed-phase HPLC mainly resulted in a thin and symmetric peak, especially in good reproducibility throughout testing: the coefficient of variation between series was 4%. The analysis of different dilutions of standard retinol allowed the establishment of a line of calibration expressing surface analytical signal in relation to vitamin concentration. The calibration curve was obtained with a very good correlation ($R = 0.99978$). Then the retinol level in each extract was determined from his corresponding peak area.

The results obtained were compared with those of earlier studies: they are recorded in Table 1.

The age and nutritional status -related variations in plasma retinol level were also studied among our population. The results in Table 2 indicate a retinol decrease in children aged from 12 to 35 months. However, our results indicate none retinol deficiency: the obtained values were normal ($>0.70\mu\text{mol/L}$). Retinol level variations were also studied according to the thick film outcomes and to the sex among the population. The results are reported in Tables 3. The analysis on account of malaria infection showed in table 3 that the retinol level is significantly lower in children with malaria compared to thick film-negative subjects ($p = 0.004$).

DISCUSSION

Malaria and vitamin A deficiency cause by their high co-prevalence and morbidity a serious public health problem in developing countries (Shankar, 1995; Marsh, 1998).

Table 1. Vitamin A status of preschool-age population in different countries (Niger)

Countries	Retinol serum status ($\mu\text{mol/L}$) $m \pm \text{ET}$	References
India	1.19 ± 0.04	(Raza et al., 2009)
China	1.65 ± 0.47	(Yang et al., 2007)
USA	1.28 ± 0.01	(Ballew et al., 2001)
Turkey	0.98 ± 0.32	(Tansu et al., 2010)
Brazilia	$1.78 \pm 0.49^*$	(Flores et al., 1991)
Gabonese	0.84 ± 0.51	(Abessolo et al. 2009)
Senegal	0.52 ± 0.03	(Carlier et al. 1992)
Sénégál	$1.75 \pm 0.98^*$	Our study

* Serum retinol status after vitamin A supplementation

Table 2. age-related variations in serum retinol levels in the study population

Age (months)	Retinol serum levels ($\mu\text{mol/L}$) $m \pm \text{ET}$
0 – 11 (n=49)	2.40 ± 1.05
12 – 23 (n=62)	$1.50 \pm 0.82^*$
24 – 35 (n=70)	$1.72 \pm 0.62^*$
36 – 59 (n=131)	2.00 ± 0.92

*significantly different from others ($p < 0.05$)

Table 3. Change in retinol plasma levels according to gender and malaria among children

Biocharacteristics	Retinol serum levels ($\mu\text{mol/L}$) $m \pm \text{ET}$
Males (n=168)	1.81 ± 1.08
Females (n=144)	$1.57 \pm 0.98^*$
Children (n=280) with negative Thick smear	1.94 ± 0.81
Children (n=32) with positive Thick smear	$1.51 \pm 0.78^{**}$

Thick smear positive: presence of plasmodium; thick smear negative: absence of plasmodium

*significantly different from males ($p < 0.05$)

**significantly different from Children with negative Thick smear ($p < 0.05$)

These two scourges form a vicious circle characterized by difficulties in the study of real impact of the carriage of Plasmodium on serum retinol status. This obstacle was overcome in our study by choosing a preschool population who had previously received vitamin A supplementation. The effectiveness of vitamin intake is confirmed by the average rate of $1.75 \pm 0.98 \mu\text{mol/L}$ as a retinol status among our study population. Therefore, to effectively uncover any negative effect of malaria on retinol level, high performance liquid chromatography or HPLC was used as assay. Indeed, the HPLC analytical method is the reference because of specificity, sensitivity and reproducibility of the results (Underwood, 1990). These analytical characteristics are confirmed in our study by the retention time reproducibility from one test to another and through symmetrical and thin peaks. They were aided by the use

of HPLC grade solvents which transmission was greater than 98% at the wavelength reading of 325nm. The good correlation obtained ($R = 0.99978$) between the analytical peak area and concentration of vitamin allowed a good determination of vitamin A in biological products of the population studied. The analysis of the results was facilitated by matching our population according to the main factors of variation in retinol levels.

The relative decline of retinol in children aged 12 to 35 months coincides with the usual weaning period for children living in rural Africa. This breastfeeding cessation is often relayed by an inadequate dietary intake (Mane et al. 2006). The decrease of retinol level may be also explained by the fast rate of growth and higher physiological requirement of retinol at that age. According to our results, the risk seems to be higher among female subjects. This variation by gender corroborates thus the

data reported by several authors (Ingenbleek et al., 1981; Olmedilla et al., 1994). In addition, the fact that our study focused retinol-sufficient children according to the OMS recommendations (retinol levels $> 0.70 \mu\text{mol/L}$) allowed survey of the real impact of malaria infection on serum levels of vitamin A. The results have unmasked a significant decrease in serum retinol concentrations among thick-film positive children compared to retinol levels of thick-negative children ($p = 0.003136$). This negative effect of malaria infection corroborates the depletion of vitamin A widely reported during malaria infection. Although, the mechanism underlying the retinol depletion during malarial infection is not fully elucidated. According to previous studies, retinol levels may fall as a consequence of inflammatory process and vitamin urinary excretion during malarial infection (Rosales et al., 1996; Miltra et al. 1998). Moreover Mizuno Y. et al. report an uptake of vitamin A by the parasite (*Plasmodium falciparum*) (Mizuno et al., 2003). These data suggest an increased utilization of vitamin A in malaria. Then they suggest a strengthening of retinol supplementation during peak malaria endemicity and more for kindergartners.

CONCLUSION

Vitamin A is an essential micro nutriment for growth and cell differentiation in general, particularly lymphocytic. Its deficiency is an aggravating factor for morbidity of infectious diseases. In this case-control study, the main results have been a relative decline in retinol linked both to gender, age and malaria infection. A high prevalence of lower retinol concentrations was observed among children with malaria. This hypovitaminosis likely induced by malaria infection constitutes thus an important argument in favor of taking corrective actions towards sustaining and strengthening the vitamin A status for children of preschool age, especially girls, and more during peaks of malaria endemicity in the rainy season. Vitamin A supplementation would be crucial in the fight against malaria which is a serious public health problem especially for preschool children living in the tropics.

ACKNOWLEDGEMENT

We thank World Vision for his precious advice in this study.

REFERENCES

- Abessolo FO, Kuissi E, Nguete JC, Zue Ndong GJL, Ngou-Milama E (2009) Vitamine A chez les enfants gabonais non supplémentés : relation avec les pathologies oculaires et nutritionnelles. *Cahier Santé*. 19: 29-33.
- Ballew C, Bowman B, Sowell AL, and Gillespie C (2001) Serum retinol distributions in residents of the United States: third National Health and Nutrition Examination Survey, 1988-1994. *Am J Clin Nutr*. 73: 586-593.
- Carlier C, Etchepare M, Ceccon JF, Amedee-Manesme O (1992) Assessment of the vitamin A status of preschool and school age Senegalese children during a cross – sectional study. *Int J Vitam Nutr Res*. 62 (3): 209 – 15.
- Das BS, Thurnham DI, Das DB (1996) Plasma α -tocopherol, retinol and carotenoids in children with falciparum malaria. *Am J Clin Nutr*. 64: 94-100.
- Flores H, Azevedo MN, Campos FA, Barreto-Lins MC, Cavalcanti AA, Salzano AC, Varela RM, Underwood BA (1991) Serum vitamin curve for children aged 2 – 6 years known to have adequate vitamin A status: a reference population. *Am J Clin Nutr*. 54 : 707 – 711.
- Hautvast JL, Tolboom JJ, West CE, Kafwembe EM, Sauerwein RW, van Staveren WA (1998); Malaria is associated with reduced serum retinol levels in rural Zambian children. *Int J Vitam Nutr Res*. 68(6):384-8.
- Ingenbleek Y, Van den Hove MF, Deruelle M (1981); Differences in the retinol circulating complex between healthy male and female infants. *Clin Chim Acta*. 114 (2 –3) : 219 24.
- Mane NB, Simondon KB, Diallo A, Marra AM, and Simondon F (2006) Early breastfeeding cessation in rural senegal: causes, modes and consequences. *Ame. J. Public Health* 96 (1): 139-144.
- Marsh K (1998); Malaria disaster in Africa. *Lancet*. 352 : 924.
- Miltra AK, Alvarez JO, Stephensen CB (1998) Increased urinary retinol loss in children with severe infections. *Lancet*. 351, 1033 – 1034.
- Mizuno Y, Kawazu SI, Kano S, Watanabe N, Matsuura T, Ohtomo H (2003) In-vitro uptake of vitamin A by *Plasmodium falciparum*. *Ann. Trop. Med. Parasitol*. 97 (3): 237-243.
- Olmedilla B, Granado F, Blanco I, Rojas-Hidalgo E (1994) Seasonal and sex-related variations in six serum carotenoids, retinol and α -tocopherol. *Ame. J. Clin. Nutr*. 60: 106-110.
- Raza A, Khan HM, Malik MA, Mahdi AA, Shahid M, Shujatullah F (2009) Serum retinol concentration in patients with acute falciparum malaria in Aligarh, India. *J infect Dev Ctries*. 3(11): 865 – 868.
- Rosales FJ, Ritter SJ, Zolfaghari R, Smith JE, Ross AC (1996) Effects of acute inflammation on plasma retinol, retinol-binding protein and its mRNA in the liver and kidneys of vitamin A-sufficient rats. *J. Lipid Res*. 37, 962 – 971.
- Sanjoaquin MA, Molyneux ME (2009) Malaria and vitamin A deficiency in African children: a vicious circle? *Malar J*. 8:134.
- Shankar AH (1995) Vitamin A and malaria. *Am J Clin Nutr*. 62: 842 – 883.
- Tansüg N, Polat M, Cesme S, Taneli F, Gözman S, Tokusoglu O, Yilmaz D, Dinç G (2010) Vitamin A status of healthy children in Manisa, Turkey. *Nutr. J*. 9: 34.
- Underwood BA (1990) Methods for assessment of vitamin A status. *J. Nutr*. 120: 1459-1463
- Yang R, Li R, Mao S, Sun L, Huang X, Ji C, Zhu Z, Wu L, Qin Y, Zhao Z (2007) The survey of serum retinol of the children aged 0-4 years in Zhejiang Province, China. *BMC Public Health*. 7: 264.